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(54) Title: UROCORTIN PROTEINS AND USES THEREOF

(57) Abstract: A human urocortin-related peptide with significant sequence homology to the CRF neuropeptide family was identi-
fied. A mouse cDNA was isolated from whole brain poly (A+) RNA that encodes a predicted 38 amino acid peptide protein designated
herein as urocortin II. Both human URP and mouse family members, CRF and urocortin (Ucn). These peptides are involved in the
regulation of the hypothalamic-pituitary-adrenal axis under basal and stress conditions, suggesting a similar role for URP and Ucn
II. Synthesized Ucn-II and URP peptide binds with higher affinity to CRF-R2 than to CRF-R1 Ucn II and human URP appear to be
involved in the regulation of body temperature and appetite and may play a role in other stress related phenomenon. These findings
identify Ucn II and human URP as a new members of the CRF family of neuropeptides, which are expressed centrally and bind to
CRF-R2.

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UROCORTIN PROTEINS AND USES THEREOF

BACKGROUND OF THE INVENTION

10

Federal Funding Legend

This invention was produced in part using funds from the Federal government under grant no. DK-26741. Accordingly, the Federal government has certain rights in this invention.

15

Field of the Invention

The present invention relates generally to the fields of neuroendocrinology and the mechanisms involved in stress. More specifically, the present invention relates to novel corticotropin releasing factor-related peptides, urocortin II and human urocortin-related protein, which are involved in the stress response.

25

Description of the Related Art

Corticotropin-releasing factor (CRF) is a 41-amino acid peptide best known for its indispensable role in initiating pituitary-adrenal responses to stress, an effect mediated by type 1 CRF receptors (1). In addition, corticotropin-releasing factor is

widely distributed in brain, and has been shown repeatedly to participate in the mobilization of complementary autonomic and behavioral adjustments to a variety of threatening circumstances (2, 3). This has fostered the widely held hypothesis that corticotropin releasing factor and its related family of peptides play important roles in regulation of the hypothalamic-pituitary-adrenal axis (HPA) under basal and stress conditions (4, 5). It is also believed that corticotropin-releasing factor is also involved in other neuroendocrine and paracrine responses in many tissues. Members of the CRF family integrate endocrine, autonomic and behavioral responses to stressors. These peptides may also be implicated in the control of appetite, arousal, and cognitive functions. Severe psychological and physiological consequences can occur as a result of the long term effects of stress, such as anxiety disorders, anorexia nervosa and melancholic depression.

Corticotropin-releasing factor family members mediate their biological actions by specifically binding to CRF receptors with high affinities (6, 7). CRF receptors are G-protein coupled receptors that act through adenylate cyclase and are structurally related to the secretin family. This family also includes GRF, VIP, PTH, and the Calcitonin receptor. The CRF receptor gene has 13 exons and several splice variants of this receptor have been found. The CRF-R1 receptor is distributed throughout the brain and is found in sensory and motor relay sites (8). The CRF-R2 α is distributed in lateral septum, ventral medial hypothalamus, nucleus of the solitary tract and the dorsal raphe nucleus, which are areas where CRF-R1 is expressed very

little or not at all (9). The CRF-R2 β is found mostly in peripheral sites including the heart, blood vessels, gastrointestinal tract, epididymis, lung and skin (7, 10). The pharmacology of the two types of receptors differs in that corticotropin-releasing factor has a low affinity for CRF-R2 (K_i =15-100 nM) but high affinity for CRF-R1 (K_i =1-2 nM). Other related peptides such as carp urotensin, frog sauvagine, and urocortin have a high affinity for CRF-R2. CRF-R2 knockout mice demonstrate an increased anxiety-like behavior caused by hypersensitivity to stressors (11).

A number of the cell groups identified as sites of peptide action in eliciting stress-like autonomic and behavioral responses have been found to be lacking or impoverished in the expression of requisite ligand(s), receptor(s) or both (12, 13). This has kindled the search for additional CRF-related signaling molecules, which currently number two ligands, G protein-coupled receptors derived from two distinct genes (CRF-R1 and CRF-R2), and a binding protein, whose function remains incompletely understood (14, 15).

A second mammalian CRF-related neuropeptide, urocortin (Ucn), was recently discovered (16) and shown to be bound with high affinity by both known CRF receptor types, whereas CRF is bound in a highly preferential manner by CRF-R1. Centrally administered urocortin is more potent than CRF in suppressing appetite but less so in generating acute anxiety-like effects and generalized behavioral activation (17). This has been taken to indicate that urocortin might mediate some stress-related effects attributed initially to CRF, at least in part by serving as an endogenous ligand for CRF-R2. This view has been

challenged, however, by such observations as that the principal cellular seats of urocortin expression in brain are not recognized as integral components of central stress-related circuitry, and that most major sites of CRF-R2 expression are poorly innervated
5 by urocortin-containing projections (18). These and other findings support the possible existence of one or more additional CRF receptor ligands in the mammalian brain.

The prior art is deficient in the lack of recognition of additional urocortin genes and proteins. The present invention
10 fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

15 The rapid advances in the deposition of sequence data for both the human and mouse genomes provide an opportunity to identify new members of many protein families. A novel peptide sequence, Human urocortin-related peptide (URP) was identified from the public human genome database. The
20 urocortin-related peptide sequence contains homology to human Urocortin (44%), carp Urotensin (39%) and human CRF (36%). The synthesized urocortin-related peptide binds with higher affinity to CRF-R2 ($K_i=0.5$ nM) than to CRF-R1 ($K_i=70$ nM). Human urocortin-related peptide stimulates ACTH secretion from
25 rat anterior pituitary cells, although with a significantly lower potency compared to urocortin or CRF. Using sequence homology searching tools, a mouse gene encoding a 38-amino-acid peptide was also identified which represents a new member

of the CRF family of neuropeptides. This peptide, termed urocortin II (Ucn II), is distinct from the other known family members in that it binds with high selectivity to CRF-R2. Evidence for Urocortin II in the rat brain is provided by
5 immunohistochemistry and *in situ* hybridization studies using antibodies highly specific for urocortin II.

In one embodiment of the current invention, a DNA sequence encoding urocortin II is provided. This sequence may be selected from the group consisting of: isolated and purified
10 DNA which encodes an urocortin II; isolated and purified DNA which hybridizes at high stringency conditions to the antisense complement of urocortin II DNA under high stringency conditions (defined as membrane washing at high temperature and low salt concentration functionally equivalent to 0.1 x SSC at
15 65°C); and, isolated and purified DNA encoding urocortin II but which differs in sequence due to the degeneracy of the genetic code. This DNA preferably encodes a protein precursor having the amino acid sequence shown in SEQ ID No: 10.

In another embodiment of the current invention, the
20 instant invention is directed to a vector capable of expressing the urocortin II. Such a vector consists of DNA encoding urocortin II and regulatory elements necessary for expression of urocortin II in a cell. In a preferred embodiment, this vector encodes a protein of amino acid sequence SEQ ID No: 11. The instant
25 invention is also directed to a host cell transfected with and expressing urocortin II from such a vector. The protein may be expressed in a cell type selected from bacterial cells, mammalian

cells, plant cells and insect cells. In one preferred embodiment, the protein is expressed in *E. coli*.

In yet another embodiment of the instant invention, an isolated and purified human urocortin II protein is provided
5 encoded from DNA as described above. Preferably, the purified human urocortin-related peptide has an amino acid sequence corresponding to SEQ ID No: 11.

In another embodiment of the instant invention, an antibody directed against the urocortin II protein is provided.
10 This antibody may be a monoclonal antibody.

In yet another embodiment of the instant invention, a pharmaceutical composition is provided comprising urocortin II protein. Such a pharmaceutical composition may be used to reduce body temperature, suppress appetite, and treat or prevent
15 congestive heart failure and various stress-related disorders.

In a further embodiment of the current invention, a DNA sequence encoding human urocortin-related peptide is provided. This sequence may be selected from the group consisting of: isolated and purified DNA which encodes an human
20 urocortin-related peptide; isolated and purified DNA which hybridizes at high stringency conditions to the antisense complement of the human urocortin-related peptide DNA under high stringency conditions (defined as membrane washing at high temperature and low salt concentration functionally
25 equivalent to 0.1 x SSC at 65°C); and, isolated and purified DNA encoding human urocortin-related peptide but which differs in sequence due to the degeneracy of the genetic code. This DNA preferably has the sequence shown in SEQ ID No: 1 and encodes a

protein precursor having the amino acid sequence shown in SEQ ID No: 2.

In another embodiment of the current invention, the instant invention is directed to a vector capable of expressing the human urocortin-related peptide. Such a vector consists of DNA encoding human urocortin-related peptide and regulatory elements necessary for expression of human urocortin-related peptide in a cell. In a preferred embodiment, this vector encodes a protein of amino acid sequence SEQ ID No: 3. The instant invention is also directed to a host cell transfected with and expressing an human urocortin-related peptide from such a vector. The protein may be expressed in a cell type selected from bacterial cells, mammalian cells, plant cells and insect cells. In one preferred embodiment, the protein is expressed in *E. coli*.

In yet another embodiment of the instant invention, an isolated and purified human urocortin-related peptide protein is provided encoded from DNA as described above. Preferably, the purified human urocortin-related peptide has an amino acid sequence corresponding to SEQ ID No: 3.

In another embodiment of the instant invention, an antibody directed against the human urocortin-related peptide protein is provided. This antibody may be a monoclonal antibody.

In yet another embodiment of the instant invention, a pharmaceutical composition is provided comprising human urocortin-related peptide protein. Such a pharmaceutical composition may be used to reduce body temperature, suppress

appetite, and treat or prevent congestive heart failure and various stress-related disorders.

In another embodiment of the instant invention, various modifications to the urocortin II and human urocortin-related peptide proteins are described including modification to the sequence and to individual amino acids of the proteins. Modifications also include conjugation of urocortin II and human urocortin-related peptide to fluorescent labels, complexed radionuclides and toxins.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others that will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows that a human genomic DNA sequence predicting the existence of a novel peptide related to urocortin and CRF. Genomic sequences were identified in the public database and used to predict the novel sequence of human urocortin-related peptide. The putative start site is at position 1

and the mature peptide sequence is shown in bold text. The predicted signal peptide cleavage sites are indicated with arrows.

Figure 2 shows a putative Human urocortin-related peptide precursor. The underlined region represents a partial
5 cDNA sequence that was isolated by PCR from a human pancreatic islet cDNA library.

Figure 3 shows a comparison of human urocortin-related peptide (URP) with human Ucn, urotensin I, CRF, frog sauvagine and dogfish CRF/Uro. Areas of highest homology are
10 inside the white boxes. The number of conserved amino acids is indicated.

Figure 4A shows the predicted amino acid sequence of Ucn II. The start methionine, marked in bold, is located upstream of the peptide coding region, which is boxed. The
15 complete nucleotide sequence has been deposited in Genbank (accession no. AF331517). Figure 4B shows the alignment of mouse Ucn II with homologous human and fish peptides (URPs) and with rat Ucn and rat/human CRF. Residues identical to the mouse Ucn II sequence are boxed. A ■ indicates an amidation
20 site.

Figure 5 shows the human urocortin-related peptide mediated displacement of ^{125}I -Sauvagine binding to CRFR1 and CRFR2 β . The affinities of Ucn and URP peptides for CRFR1 and CRFR2 β stably expressed in CHO cells were determined by the
25 competitive displacement of the ^{125}I -Sauvagine. The data is representative of 3 experiments and inhibitory dissociation constant (K_i) values (95% confidence limits) were calculated using the Prism program.

Figures 6A-6C show urocortin II mRNA expression in the rat brain. Darkfield photomicrographs showing labeling (white grains) observed over select regions using an isotopically labeled antisense cRNA probe generated from a mouse urocortin II cDNA. Positive hybridization signals are seen over the paraventricular nucleus of the hypothalamus (Figure 6A), principally over its magnocellular division (pm), with more diffuse signal seen over the parvocellular aspect (mp), and broadly over the locus coeruleus (LC; Figure 6B), facial motor nucleus (VII, Figure 6C) and meninges (men) at the ventral surface of the brain. Other abbreviations: CBL, cerebellum; v3, third ventricle; v4, fourth ventricle. Magnifications: Figures 6A and 6B, X75; Figure 6C, X50.

Figure 7 shows an autoradiogram of human urocortin-related peptide expression in the primate hypothalamus. PVH, paraventricular nuclei; SO, supraoptic nuclei; CN, caudate nucleus; och, optic chiasm; me, median eminence; ac, anterior commissure; ic, internal capsule; Sept, septum.

Figures 8A-8F show cellular activation patterns in response to central urocortin II microinjection. Figures 8A-8C and 8E: Brightfield photomicrographs of immunoperoxidase preparations showing induced Fos expression in rats sacrificed 2 hr after icv injection of 1 μ g synthetic mouse urocortin II. Darkfield photomicrographs showing hybridization histochemical localization of CRF-R2 mRNA in regions corresponding to those illustrated in Figures 8C and 8E are provided in Figures 8D and 8F, respectively. Central urocortin II injection provoked Fos

induction primarily in a set of interconnected structures involved in central autonomic and neuroendocrine control, including the parvocellular division of the paraventricular nucleus (Figure 8A), the central nucleus of the amygdala, (Figure 8B), and the nucleus of the solitary tract (NTS, Figure 8C). Among these, only the NTS is a site of CRF-R2 expression (Figure 8D). Other principal sites of CRF-R2 expression, including the ventromedial nucleus of the hypothalamus (Figure 8F), failed to show urocortin II-induced Fos expression over the range of peptide doses examined (1-10 μ g). All photomicrographs are of 75X magnification.

Figure 9 shows the activation of central stress-related cell groups following central injection of human urocortin-related peptide by examining stimulation of nuclear FOS expression in the stria terminalis (BST), paraventricular nucleus of the hypothalamus (PVH), central nucleus of the amygdala (CeA), the lateral parabrachial nucleus (PBI), the locus coeruleus (LC) and the nucleus of the solitary tract (NTS). BSTov, bed nucleus of the stria terminalis (oval subnucleus); ic, internal capsule; CP, caudoputamen; ac, anterior commissure; V3, third ventricle; AHA, anterior hypothalamic area; pm, posterior magnocellular part (paraventricular nucleus); fx, fornix; CeAm, central nucleus of the amygdala (medial part); BLA, basolateral nucleus of the amygdala; scp, superior cerebellar peduncle; PBel, parabrachial nucleus (external lateral part); V4, fourth ventricle; ep, ependyma; AP, area postrema; DMX, dorsal motor nucleus of the vagus; ts, solitary tract; and, cc, central canal.

Figures 10A and 10B show the effects of central urocortin II on food intake and gross motor activity. Figure 10A shows the mean (\pm SEM; n=3-6 per group) cumulative nighttime food intake (g) following icv administration of 1 μ g CRF, urocortin or urocortin II. Both CRF and urocortin significantly reduced food intake compared to saline-injected controls, beginning at 4 hr post-injection, while the effect of urocortin II was not manifest until 6 hr after treatment. * p <.002 (CRF and Ucn vs. saline), ** p <.002 (CRF, urocortin, and urocortin II vs. saline). Figure 10B shows telemetric measures of gross motor activity which were significantly elevated in animals that received icv injections of CRF; neither urocortin nor urocortin II significantly affected motor activity. * p <.001 (CRF vs. saline).

Figure 11 shows stimulation of ACTH secretion from rat anterior pituitary cells by urocortin and human urocortin-related peptide. Rat anterior pituitary cells were established in culture and treated with either rat urocortin or human urocortin-related peptide. Secreted ACTH was measured using a kit (Nichols Institute Diagnostics).

Figure 12 shows the effect of human urocortin-related peptide on cAMP levels in A7R5 cells, which express native CRF-R2 β . Dose-dependent effects of incubation with urocortin (open circle) or hURP (solid circle) for 30 minutes on cAMP production. cAMP was measured by RIA (Biochemical Technologies).

Figure 13 shows the effects of human urocortin-related peptide (hURP) on gross motor activity in rats.

Figure 14 shows the effects of intracerebroventricular injection of human urocortin-related peptide (URP) on body temperature in rats.

Figure 15 shows the effects of intracerebroventricular injection of human urocortin-related peptide (hURP) on nocturnal food intake in rats.

Figure 16 show a model of how human urocortin-related peptide acts on CRF-R1 and CRF-R2. Human urocortin-related peptide binds with high affinity to CRF-R2 but not CRF-R1 while urocortin binds to both receptors. CRF binds with high affinity to CRF-R1 and not to CRF-R2.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins Eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins Eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

As used herein, the term "cDNA" shall refer to the DNA copy of the mRNA transcript of a gene.

5 As used herein, the term "derived amino acid sequence" shall mean the amino acid sequence determined by reading the triplet sequence of nucleotide bases in the cDNA.

As used herein the term "screening a library" shall refer to the process of using a labeled probe to check whether,
10 under the appropriate conditions, there is a sequence complementary to the probe present in a particular DNA library. In addition, "screening a library" could be performed by PCR.

As used herein, the term "PCR" refers to the polymerase chain reaction that is the subject of U.S. Patent Nos.
15 4,683,195 and 4,683,202 to Mullis, as well as other improvements now known in the art.

All amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus.
20 Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues.

The amino acids described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric
25 form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin binding is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to

the free carboxy group present at the carboxy terminus of a polypeptide.

Nonstandard amino acids may be incorporated into proteins by chemical modification of existing amino acids or by
5 artificial synthesis of a protein. A Nonstandard amino acid refers to an amino acid that differs in chemical structure from the twenty standard amino acids encoded by the genetic code. Post-translational modification in vivo can also lead to the presence of a nonstandard or amino acid derivative in a protein. The N-
10 terminal NH_2 and C-terminal COOH groups of a protein can also be modified by natural or artificial post-translational modification of a protein.

Proteins may be modified by amino acids substitutions. Often, some changes result in significant changes
15 in the activity of proteins while other have little or no effect. Conservative substitutions are least likely to drastically alter the activity of a protein. A "conservative amino acid substitution" refers to replacement of amino acid with a chemically similar amino acid, i.e. replacing nonpolar amino acids with other
20 nonpolar amino acids; substitution of polar amino acids with other polar amino acids, acidic residues with other acidic amino acids, etc., Examples of preferred conservative substitutions are set forth in Table I:

TABLE I

Original Residue	Preferred Conservative Substitutions	Most Preferred Conservative Substitution
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Lys; Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Nle	Leu
Leu (L)	Ile; Val; Met; Ala; Phe; Nle	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Leu; Val; Ile; Ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Nle	Leu

"Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized polypeptides include, for example, those in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butylocycarbonyl groups, chloroacetyl groups, or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Chemical derivatives may include those peptides which contain one or more naturally occurring amino acids derivatives of the twenty standard amino acids. For example, 4-hydroxyproline may be substituted for serine; and ornithine may be substituted for lysine. Peptides embraced by the present invention also include peptides having one or more residue additions and/or deletions relative to the specific peptide whose sequence is shown herein, so long as the modified peptide maintains the requisite biological activity.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This

term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

A DNA "coding sequence" is a double-stranded DNA sequence, which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating

transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the
5 minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic
10 promoters often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of
15 another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

20 A "signal sequence" can be included near the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, which communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell
25 before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

The term "oligonucleotide", as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact

sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementary with the sequence or hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90% or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that
5 are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within
10 the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature.
15 Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where
20 the genomic coding sequence contains introns or synthetic sequences having codons different than the native gene). Allelic variations or naturally occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

The labels most commonly employed for these studies
25 are radioactive elements, enzymes, chemicals that fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA

blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

A particular assay system developed and utilized in the art is known as a receptor assay. In a receptor assay, the material to be assayed is appropriately labeled and then certain cellular test colonies are inoculated with a quantity of both the label after which binding studies are conducted to determine the extent to which the labeled material binds to the cell receptors. In this way, differences in affinity between materials can be ascertained.

As used herein, the term "host" is meant to include not only prokaryotes but also eukaryotes such as yeast, plant and animal cells. A recombinant DNA molecule or gene that encodes a protein of the present invention can be used to transform a host using any of the techniques commonly known to those of ordinary skill in the art. Prokaryotic hosts may include *E. coli*, *S. typhimurium*, *Serratia marcescens* and *Bacillus subtilis*. Eukaryotic hosts include yeasts such as *Pichia pastoris*, mammalian cells and insect cells.

In general, expression vectors containing promoter sequences that facilitate the efficient transcription of the inserted DNA fragment are used in connection with the host. The expression vector typically contains an origin of replication, promoter(s), terminator(s), as well as specific genes that are capable of providing phenotypic selection in transformed cells. The transformed hosts can be fermented and cultured according to means known in the art to achieve optimal cell growth.

Methods well known to those skilled in the art can be used to construct expression vectors containing appropriate transcriptional and translational control signals. See for example, the techniques described in Sambrook et al., 1989, 5 *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Press, N.Y. A gene and its transcription control sequences are defined as being "operably linked" if the transcription control sequences effectively control the transcription of the gene. Vectors of the invention include, but 10 are not limited to, plasmid vectors and viral vectors.

The current invention is directed to a DNA sequence encoding urocortin II. This sequence may be an isolated and purified DNA that encodes an urocortin II. Alternatively, it may be an isolated and purified DNA which hybridizes at high 15 stringency conditions to the antisense complement of the urocortin II DNA under high stringency conditions (defined as membrane washing at high temperature and low salt concentration functionally equivalent to 0.1 x SSC at 65°C). Finally, the DNA may be an isolated and purified DNA encoding 20 urocortin II but which differs in sequence due to the degeneracy of the genetic code. This DNA will preferably encode a protein of amino acid sequence SEQ ID No: 10 or amino acid SEQ ID No. 11.

The instant invention is also directed to a vector capable of expressing the urocortin II. Such a vector consists of 25 DNA encoding urocortin II and regulatory elements necessary for expression of urocortin II in a cell. In a preferred embodiment, this vector encodes a protein of amino acid sequence SEQ ID No: 10 or amino acid SEQ ID No. 11. The instant invention is also

directed to a host cell transfected with and expressing an urocortin II from such a vector. The protein may be expressed in a cell type selected from bacterial cells, mammalian cells, plant cells and insect cells. In a preferred embodiment, the protein is
5 expressed in *E. coli*.

The instant invention is also directed to an isolated and purified urocortin II protein encoded from DNA as described above. Preferably, the purified urocortin II has an amino acid sequence corresponding to SEQ ID No: 10 or SEQ ID No. 11.

10 The instant invention is also directed to an antibody directed against the urocortin II protein. This antibody is preferably a monoclonal antibody.

Furthermore, the instant invention is directed to a pharmaceutical composition comprising the urocortin II protein
15 and a pharmaceutically acceptable carrier. Such a pharmaceutical composition may be used to reduce body temperature, suppress appetite, treat or prevent congestive heart failure, treat stress and anxiety, and alter undesirably low levels of ACTH secretion.

20 The current invention is also directed to a DNA sequence encoding human urocortin-related peptide. This sequence may be an isolated and purified DNA that encodes human urocortin-related peptide. Alternatively, it may be an isolated and purified DNA which hybridizes at high stringency
25 conditions to the antisense complement of the human urocortin-related peptide DNA under high stringency conditions (defined as membrane washing at high temperature and low salt concentration functionally equivalent to 0.1 x SSC at 65°C).

Finally, the DNA may be an isolated and purified DNA encoding human urocortin-related peptide but which differs in sequence due to the degeneracy of the genetic code. This DNA will preferably have the sequence shown in SEQ ID No: 1 and will preferably encode a precursor protein of amino acid sequence SEQ ID No: 2 which is proteolytically processed to a protein of amino acid sequence SEQ ID No. 3.

The instant invention is also directed to a vector capable of expressing the human urocortin-related peptide. Such a vector consists of DNA encoding human urocortin-related peptide and regulatory elements necessary for expression of human urocortin-related peptide in a cell. In a preferred embodiment, this vector encodes a protein of amino acid sequence SEQ ID No: 3. The instant invention is also directed to a host cell transfected with and expressing human urocortin-related peptide from such a vector. The protein may be expressed in a cell type selected from bacterial cells, mammalian cells, plant cells and insect cells. In a preferred embodiment, the protein is expressed in *E. coli*.

The instant invention is also directed to an isolated and purified human urocortin-related peptide protein is provided encoded from DNA as described above. Preferably, the purified human urocortin-related peptide has an amino acid sequence corresponding to SEQ ID No: 3.

The instant invention is also directed to an antibody directed against the human urocortin-related peptide protein is provided. This antibody is preferably a monoclonal antibody.

Furthermore, the instant invention is directed to a pharmaceutical composition comprising the human urocortin-related peptide protein and a pharmaceutically acceptable carrier. Such a pharmaceutical composition may be used to
5 reduce body temperature, suppress appetite, treat or prevent congestive heart failure, treat stress and anxiety, and alter undesirably low levels of ACTH secretion.

The instant invention is also directed to urocortin II or human urocortin-related peptide mutated to contain a
10 tyrosine residue, which for radioiodination of the protein. One particular modification is the addition of a sequence consisting of Tyr-Gly to the N-terminal end of urocortin II or human urocortin-related peptide.

The instant invention is also directed to deletion
15 mutants of urocortin II or human urocortin-related peptide. A particularly useful deletion is a deletion of one to five amino acids from the N-terminal end of the protein.

The instant invention is also directed to urocortin II or human urocortin-related peptide protein in which the
20 standard "L-form" isomeric amino acids are replaced with "D-form" isomeric amino acids. In human urocortin-related protein, substitution of the isoleucine residue corresponding to position 9 of SEQ ID No. 3 with D-isoleucine, D-phenylalanine, and D-Leucine or other D-form amino acids is particularly useful.
25 Another useful substitution is the replacement of the glutamic acid residue at position 17 of SEQ ID 3 or 11 with D-glutamic acid.

The instant invention is also directed to urocortin II or human urocortin-related peptide in which various amino acids have been replaced with nonstandard amino acids. C α -methylated leucine, C α -methylated alanine, N-im-benzylhistidine, 4-hydroxyproline, 5-hydroxylysine, 3-methylhistidine, homoserine, and ornithine are examples of such nonstandard amino acids.

The instant invention is also directed to urocortin II or human urocortin-related peptide protein having an acylated N-terminus. This protein acylation may be used to link a molecule such as fatty acid at the N-terminus of the protein to protect Ucn II or URP from enzymatic degradation or to change various properties of the protein such as its hydrophilicity/hydrophobicity. These modification may be used alter the duration or bioavailability of the protein *in vivo*.

The instant invention is also directed to urocortin II or human urocortin-related peptide protein that has been modified to contain a fluorescent label for use in imaging or biological assays.

The instant invention is also directed to a urocortin II or human urocortin-related peptide protein conjugated with a complexing agent for radionuclides. Ucn II complexed to a radionuclide may be useful for scintigraphy or in various assays.

The instant invention is also directed to urocortin II or human urocortin-related peptide conjugated to a toxin. The resulting toxic conjugate can be used for the targeted destruction of CRF receptor-bearing cells.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

5

EXAMPLE 1

Identification of human urocortin-related protein

In an effort to identify novel CRF-R ligands, a hidden Markov model (HMM) was constructed from a clustal W alignment of known CRF family proteins, including rat/human CRF, rat Ucn, human Ucn, frog sauvagine, and white-suckerfish urotensin I, using the HMMER software package (Sean Eddy, Department of Genetics, Washington University, St. Louis, MO; see ref. 19). This HMM was used to search the public human genome database and a BAC (Genbank accession no. AC005903) derived from chromosome 3p21.3-4 was identified that contained a 109 bp region exhibiting significant sequence homology but which was not a part of a previously identified gene. This region was extended to 621 bp with the identification of a human EST clone that overlapped with this sequence (Genbank accession No. BE622276). The human sequence, however, lacks a consensus proteolytic cleavage site that would allow for C-terminal processing of the peptide. Therefore, the protein was designated as a human urocortin-related peptide (hURP) sequence. Figure 1 shows the nucleotide (SEQ ID No. 1) sequence of the predicted open reading frame of the human URP protein. This gene encodes a peptide of amino acid sequence SEQ ID No. 2.

To confirm the existence and sequence of the human urocortin-related peptide gene, oligonucleotide primers similar to those used to amplify the human urocortin-related peptide sequence from the genomic clone were used to isolate a partial
5 cDNA fragment by PCR from a human pancreatic islet cDNA library. This fragment was also subcloned into pGEM vector and sequenced. The sequence of the cDNA corresponded to a portion of the genomic sequence. The partial cDNA sequence corresponds to the underlined sequence in Figure 2. The
10 sequences shown in Figures 1 and 2 encode a polypeptide precursor of the human urocortin-related peptide. The first 19 nucleotides of human urocortin-related peptide encode a signal peptide that is cleaved during the post-translational modification of the protein to yield mature human urocortin-related peptide
15 of amino acid sequence:

I V L S L D V P I G L L Q I L L E Q A R A R A A R E Q A T
T N A R I L A R V G H C-NH₂ (SEQ ID No. 3).

20

Figure 3 shows the results of a homology comparison between amino acids 72-109 of human urocortin-related peptide and equivalent segments of human urocortin, human urotensin I, human corticotrophin releasing factor (CRF), frog sauvagine, and
25 dogfish CRF/Uro. The homology in this region ranges from 26% to 42%.

EXAMPLE 2

Identification of Mouse Urocortin II

Fragmentary cDNA probes based on the human gene sequence specifically cross-hybridized with rat tissue (brain),
5 suggesting that a reasonable degree of homology existed between the two species. Based on this human sequence, primers were designed to identify the homologous mouse gene by the Rapid Amplification of cDNA Ends (RACE) method. RACE -ready cDNA was prepared from mouse whole-brain poly(A+) RNA using the
10 SMART RACE cDNA amplification kit (Clontech). PCR reactions were run under low stringency (low T_m) conditions in an effort to allow for the maximal heterologous priming. First round amplification was carried out using a touchdown protocol (94°, 30 sec; increment from 70° to 55°, 30 sec; 72°, 3 min) followed
15 by a second round of amplification with multiple sets of nested primers (94°, 20 sec; 55°, 20 sec; 72°, 3 min). Candidate PCR products were cloned into pCRII-TOPO (Invitrogen) for sequencing of both strands. Candidate 5' and 3' reaction products were identified based on their predicted size (deduced
20 from the human sequence), cloned and sequenced.

The predicted amino acid sequence for the mouse Ucn II is listed in Fig. 4A. The gene encodes a 112 amino acid precursor, and the C-terminus includes the coding region for the putative 38 amino acid mature peptide, indicated in the boxed
25 region (Fig. 4A). The C-terminal portion of the coding sequence is followed by a glycine and paired basic residues (R-R), presumed to be involved in amidation and cleavage from the precursor, respectively.

Two other putative or known urocortin-related peptides exist: the human one, whose peptide sequence was deduced from the published human EST, as well as a recently cloned (20) pufferfish URP (from *Takifugu rubripes*). Alignment with the human and fish urocortin-related peptides, rat Ucn, and rat/human CRF is shown in Fig. 4B. At the amino acid level, the coding region of mouse Ucn II displays 77% and 45% homology with the human and fish urocortin-related peptides, respectively. Mouse Ucn II is comparably related to known members of this peptide family, sharing 36% and 44% amino acid identity with rat CRF and rat UCN, respectively. Allowing for conservative substitutions, relatedness increases to 62% (with CRF) and 59% (Ucn).

EXAMPLE 3

Peptide synthesis

Murine Ucn II and human Ucn-related peptide were synthesized manually using the solid phase approach, a methylbenzhydryl amine resin and the Boc-strategy (21). Trifluoroacetic acid, 60% in dichloromethane, was used to remove the Boc group. Main chain assembly was mediated by diisopropylcarbodiimide. The peptides were cleaved and deprotected in hydrofluoric acid and purified using RP-HPLC and three solvent systems (triethylammonium phosphate at pH 2.25 and 6.5 and/or 0.1% TFA) (22). Peptides were greater than 95% pure using independent HPLC and CZE criteria. Mass spectra was used to confirm the composition of the preparations.

EXAMPLE 4

Receptor activation by Ucn II

The affinity of Ucn II to the CRF-R1 and CRF-R2 receptors was evaluated using a radioreceptor assay. Crude
5 membrane fractions were prepared from CHO cells stably expressing either cloned CRF-R1 or CRF-R2 β . Test peptides and the radioligand, ^{125}I -[Tyr⁰,Glu¹,Nle¹⁷]-sauvagine, were diluted in assay buffer (20 mM HEPES, 2 mM EGTA, 0.1% BSA, 10% sucrose, pH 7.6) and combined with the receptor membrane
10 preparations in MAGV microtiter plates (Millipore) pre-coated with 0.1% polyethylene imine. The reaction mixture was incubated for 90 min at room temperature followed by rapid washing twice with assay buffer and filtration. The radioligand complex was quantified by gamma radiation counting. Inhibitory
15 binding constants were determined using Prism software. The results are summarized in Table 2.

TABLE 2

5 Binding properties and functional activities
of select CRF receptor ligands

Peptide	CRF-R1		CRF-R2	
	Avg. K_i (nM) (Binding)	Avg. EC_{50} (nM) (cAMP)	Avg. K_i (nM) (Binding)	Avg. EC_{50} (nM) (cAMP)
Urocortin II (mouse)	>100	>100	0.66 (0.13-3.3)	0.14 (0.03-0.52)
URP (human)	>100	>100	0.50 (0.22-1.16)	0.42 (0.16-1.1)
Urocortin (rat)	0.32 (0.14-0.77)	0.29 (0.12-0.70)	0.62 (0.14-2.8)	0.17 (0.043-0.68)
Sauvagine (frog)	0.94 (0.49-1.8)	N/A	1.7 (0.77-3.9)	N/A

The values were determined from 3-6 independent experiments using stably transfected CHO cells or their membranes for each
 10 test peptide. EC_{50} and K_i values were determined using Prism software. Their \log_{10} values were averaged (γ). The average EC_{50} or K_i was taken to be 10^γ . The standard deviation of the \log_{10} values was calculated (σ). The ranges given were taken to be: $[(10^\gamma)10^\sigma \text{ or } 10^\gamma/10^\sigma]$.

Compared to urocortin, Ucn II was at least 1000-fold less effective at competing for binding of labeled sauvagine to the CRF-R1 whereas it was nearly equipotent to Ucn in competing for binding to CRF-R2. This significant selectivity for the type 2
5 receptor was seen also in receptor activation as measured by accumulation of intracellular cAMP. Stably transfected CHO cells (cultured in DMEM/10% FBS) were plated into 48-well tissue culture dishes (Costar) and allowed to recover for 24 hours. The medium was changed to DMEM/0.1% FBS at least two hours
10 before treatment. The cells were preincubated for 30 min' with 0.1 mM 3-isobutyl-1-methylxanthine and then exposed to peptides for 20 min at 37°C. Intracellular cAMP was extracted and measured in duplicate from triplicate wells using a RIA kit (Biomedical Technologies). In the cAMP assay, Ucn II displayed a
15 comparable efficacy for CRF-R2 as did Ucn (Table 2). The extremely low affinity of Ucn II for CRF-R1 precluded a determination of its efficacy on this receptor.

EXAMPLE 5

20 Binding was performed in 96-well 0.2 μ m Durapore plates using the vacuum filtration multiscreen assay system (Millipore). Each well contained a total volume of 200 μ l consisting of 50 μ l of binding buffer (10 % Sucrose, 0.1% BSA, 2 mM EGTA, 20 mM HEPES buffer, pH 7.5); 50 μ l of unlabeled competitor (urocortin
25 or human urocortin-related peptide) at various dilutions in binding buffer; 50 μ l 125 I-Sauvagine at a concentration of 150,000 cpm/well; and, 50 μ l of cell membranes. Plates were incubated for 1 hour at room temperature, vacuum-filtered, washed twice

with binding buffer, and allowed to dry. Individual filters were punched out and counted using a gamma counter.

Human urocortin-related peptide mediated displacement of ^{125}I -Sauvagine binding to CRFR1 and CRFR2 β stably expressed in CHO cells is shown in Figure 5. From this data, human urocortin-related peptide was found to have a disassociation constant (K_d) of 78 nM for CRF-R1 and 0.23 nM for CRF-R2 β . Ucn, on the other hand, had a disassociation constant of 0.13 nM for CRF-R1 and 0.15 nM for CRF-R2 β . Therefore, human urocortin-related peptide is much more specific for the corticotropin releasing factor type II receptor than urocortin.

EXAMPLE 6

Ucn II mRNA expression

Hybridization histochemistry was carried out to analyze the pattern of Ucn II mRNA expression in mouse and rat brain. Animals were deeply anesthetized with chloral hydrate (350 mg/kg, ip) and perfused via the ascending aorta with saline followed by ice-cold 4% paraformaldehyde in 0.1% borate buffer pH 9.5. Brains were postfixated for 16 hr and cryoprotected overnight in 10% sucrose in 0.1M phosphate buffer. Four (mice) or six (rats) series of 30 μm -thick frozen sections were cut using a sliding microtome, collected in cold ethylene glycol-based cryoprotectant and stored at -20°C until histochemical processing.

In situ hybridization was performed using ^{35}S -labeled antisense and sense (control) cRNA probes (23), constructed by

first linearizing the TOPO-II plasmid containing the mouse cDNA. Probes were labeled to specific activities of $1-3 \times 10^9$ dpm/ μ g, applied to slides at concentrations of about 10^7 cpm/ml and hybridized overnight at 56°C under high stringency (50% formamide). Final washes were carried out in 15 mM NaCl/1.5 mM sodium citrate at $65-68^\circ\text{C}$. Slides were then dehydrated and exposed to x-ray film (β -Max; Kodak) for 16 hr and then coated with Kodak NTB-2 liquid emulsion and exposed at 4°C for 21-28 days.

Hybridization histochemistry revealed a consistent and restricted pattern of Ucn II mRNA expression in mouse and rat brain. Sense-strand runoffs labeled to similar specific activities as antisense probes failed to yield above-background hybridization signals. The observed distribution of Ucn II mRNA was seen to be predominantly subcortical, with major sites of expression including stress-related cell groups such as the paraventricular, supraoptic and arcuate nuclei of the hypothalamus, and the locus coeruleus of the rostral pons (Fig. 6). Motor nuclei of the brainstem (trigeminal, facial, hypoglossal), as well of the spinal ventral horn, were also identified as sites of Ucn II mRNA expression. Among non-neuronal elements, positive hybridization signals were observed consistently over the meninges, but not the choroid plexus or ependyma. No clear suggestion of Ucn II mRNA expression by glial elements was evident.

EXAMPLE 7

Urocortin-related peptide expression in the primate brain

The expression of human urocortin-related peptide in the primate brain was examined by *in situ* hybridization. The *in situ* hybridization was performed on sections of brain tissue from *Macaca fascicularis* using an ³⁵S-labeled antisense cRNA probe corresponding to about 400 base pairs of human urocortin-related peptide. The probe was applied to the slide at a concentration of 10⁷ cpm/ml and hybridization was allowed to proceed overnight. After hybridization, the slide were treated with 20 µg/ml of ribonuclease A for 30 minutes at 37°C and washed in 15 nM NaCl/1.5 mM sodium citrate/50% formamide at 70°C. Slides were dehydrated and exposed to X-ray film (BetaMax; Kodak) for 24 hours. A sample autoradiogram is shown in Figure 7. Positive signal for URP is observed in the paraventricular (PVH) and supraoptic nuclei of the primate hypothalamus.

EXAMPLE 8

Ucn II-induced Fos expression

To identify cell groups responsive to central Ucn II administration, and to evaluate the extent to which these may conform to sites of CRF-R2 expression, the induced expression of the immediate-early gene product, Fos, in response to icv peptide administration was monitored. Adult male Sprague-Dawley rats (250-300g at start of experiments) and C57 BL/6 mice (25-40g) were housed in a colony room on a 12:12 light:dark cycle, and

with free access to food and water prior to experimentation. For intracerebroventricular (icv) injections, rats were anesthetized with ketamine/xylazine/acepromazine and stereotactically implanted with a 26 ga guide cannula terminating in the lateral
5 ventricle. For intravenous (iv) administration of peptides, animals were fitted with indwelling jugular venous catheters.

Rats that received icv injections were also implanted intra-abdominally with a transmitter to remotely monitor gross activity levels and body temperature (Mini-Mitter). After surgery,
10 animals were allowed to recover for 7 days prior to any experimentation, during which time they were handled daily. All procedures were approved by the Institutional Animal Care and Use Committee of the Salk Institute.

To monitor induced patterns of Fos expression, rats
15 were injected at 10am, either icv or iv with synthetic Ucn II (1, 5 or 10 µg/animal in 2 µl saline for icv injections or 200 µl for iv administration), or vehicle alone, and perfused two hours later. To monitor the effect of peptide administration on food intake, animals were injected icv with synthetic mouse Ucn II, rat Ucn, or
20 rat/human CRF 30 min prior to lights out. Consumption was then measured hourly for 6 hrs and at 12 hrs. Data were analyzed using repeated measures analysis of variance (ANOVA), with the Bonferoni correction for multiple comparisons applied as warranted.

25 For immunohistochemistry, tissue was pretreated sequentially with 0.3% hydrogen peroxide and 1% sodium borohydride. It was then permeabilized with PBS/0.2% triton X-100, and incubated with primary antiserum for 48 hr in PBS/2%

blocking serum. Fos immunoreactivity was localized using a polyclonal antiserum raised in rabbit against an N-terminal synthetic fragment of human Fos protein (Santa Cruz Biotechnology, 1:5K). Localization was performed using a
5 conventional avidin-biotin immunoperoxidase method with nickel enhancement, as described (24).

Injection of 1 μ g synthetic Ucn II gave rise to activational responses that were most salient in a group of interconnected structures involved in central autonomic control (25, 26). These included discrete aspects of the bed nucleus of the stria terminalis, the central nucleus of the amygdala, the paraventricular nucleus of the hypothalamus (PVH), parabrachial nucleus and nucleus of the solitary tract (NTS; Fig. 8). Of these, only the NTS has been described as a locus of CRF-R2 expression (27). Fos induction in other major sites of CRF-R2 expression, including the lateral septum, midbrain raphe nuclei and the ventromedial nucleus of the hypothalamus (27, 28), was not distinguishable from that seen in saline-injected controls. Higher doses of peptide (5 or 10 μ g) provoked more robust activational responses of similar distribution.

To control for potential systemic effects of icv injections, a similar range of Ucn II doses was given intravenously to separate groups of rats. Only the highest (10 μ g) dose gave rise to Fos induction that was clearly above control levels. Although the pattern was similar to that seen in response to central injections, neither the number of labeled cells nor their staining intensity approached that seen reliably following icv injections of 1 μ g Ucn II.

EXAMPLE 9

25 Urocortin-related peptide stimulated FOS expression in the brain

Activation of central stress-related cells groups by human urocortin-related peptide was examined by detection of the Fos gene product in the cells following injection with human

urocortin-related peptide. Rats were implanted with guide cannulae in a lateral cerebral ventricle seven days prior to experimentation. On the day of testing, the rats were injected with 5 µg synthetic human urocortin-related peptide in 5 µl of sterile saline. The rats were sacrificed two hours later and slides of various brain sections were prepared. The slides were stained by immunoperoxidase localization of Fos-immunoreactivity using a polyclonal serum raised in rabbit against residues 3-16 of the human Fos protein.

As shown in Figure 9, Fos-immunoreactivity was detected in the bed nucleus of the stria terminalis (BST), the paraventricular nucleus of the hypothalamus (PVH), the central nucleus of the amygdala (CeA), the lateral parabrachial nucleus (PBI), the locus coeruleus (LC), and nucleus of the solitary tract (NTS). Each of these sites has been previously implicated as a site of CRF-related peptide activity.

EXAMPLE 10

Behavioral effects of Ucn II

Like CRF and Ucn, Ucn II is also capable of acting centrally to inhibit food intake (Fig. 10A). Measures from separate groups of rats injected with these peptides (1 µg, icv) at the beginning of the nocturnal phase of their day-night cycle manifest a significant interaction between treatment and time point [$F(18,95)=4.22$, $p<.0001$], with both main effects also achieving reliability. All three peptides significantly reduced food intake over the 12 hr interval, with the degree of suppression ranging from 30% (CRF) to 35% (Ucn II) to 70%

(Ucn). These effects tended to be distributed differentially over time, with both Ucn- and CRF-treated animals eating significantly less than saline-injected controls earlier in the test period (4-5 hr) than did Ucn II-treated rats (6 hr).

5 In these same subjects, gross motor activity and body temperature were monitored telemetrically (Fig. 10B). Analysis of activity data revealed a significant interaction between drug and time point [$F(33,110)=1.94$, $p<.006$], with both main effects also achieving significance. Post-hoc comparisons revealed that
10 animals that received CRF were significantly more active than vehicle-treated rats over the interval 2-6 hrs post-injection ($p<.001$). Neither Ucn nor Ucn II treatment provoked reliable alterations in this measure at any post-injection time point. Core body temperature was also recorded, with each peptide
15 provoking comparably mild ($0.5-1^{\circ}\text{C}$) and transient (2 hr) hypothermic responses (data not shown).

EXAMPLE 11

20 In vitro bioassay of hURP-mediated effects on rat anterior pituitary cells

For pituitary actions, ACTH secretion response to human urocortin-related peptide was measured in primary cultures of rat anterior pituitary cells as described (30). ACTH levels were determined using the ACTH immunoassay kit from
25 Nichols Institute Diagnostics. The rat anterior pituitary cells were treated with either rat urocortin or human urocortin-related peptide and the level of secreted ACTH was measured using a kit (Nichols Institute Diagnostics). The effects of urocortin and

human urocortin-related peptide on ACTH secretion are shown in Figure 11. The stimulation of ACTH secretion in anterior pituitary cells was found to be less sensitive to human urocortin-related peptide than to urocortin.

5

EXAMPLE 12

In vitro Bioassay of the effects of hURP on A7R5 cells

The effect of hURP on cAMP levels in A7R5 cells which
10 express native CRF-R2 β was determined. The A7R5 cell line was maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 μ g/ml Streptomycin. Cells were seeded at 10,000 cells/cm² and grown for six days. Serum starved cells were preincubated with 0.1 mM 3-isobutyl-1-methylxanthine in
15 assay medium for 20 minutes and treated with the indicated concentrations of peptide for 30 minutes. cAMP levels were measured by RIA (Biochemical Technologies) and are shown in Figure 12. Human urocortin-related peptide has similar effects on cAMP production as urocortin.

20

EXAMPLE 13

Effects of human urocortin-related peptide on overall activity

To determine if human urocortin-related peptide plays a role in the generation of stress response, the effect of
25 human urocortin-related peptide on the gross motor activity of rats was examined. Cannulas were surgically into the right lateral ventricle while telemeters were implanted intraabdominally to allow for continuous monitoring of gross motor activity. The

animals were allowed a post-surgical recovery period of seven days. During the time, the animals were handled daily to acclimatize the animals to the injection procedure. On the day of injection, baseline activity was for recorded for four hours. At 5 6:00 p.m., which was the onset of lights out, the animals received an injection of either 5 μ l of saline or 5 μ l of saline containing a total of 5 μ g of human urocortin-related peptide. Activity counts were summated over a four hour time period. The results are summarized in Figure 13. No significant difference in gross 10 motor activity was seen in human urocortin-related peptide injected animals as compared to control animals.

EXAMPLE 14

Effects of human urocortin-related peptide on body temperature

15 The effect of human urocortin-related peptide on the body temperature of rats was examined. Cannulas for injection of human urocortin-related peptide were surgically into the right lateral ventricle. Telemeters for the continuous unobtrusive analysis of body temperature were implanted intraabdominally. 20 The animals were allowed a post-surgical recovery period of seven days. During the time, the animals were handled daily to acclimatize the animals to the injection procedure. On the day of injection, baseline temperature was for recorded for three hours. At 6:00 p.m. (the onset of lights out) the animals were injected 25 with either 5 μ l of saline or 5 μ l of 1 μ g/ μ l human urocortin-related peptide in saline. Body temperature was monitored every five minutes for twelve hours. As seen in Figure 14, human urocortin-related peptide injected animals had lower body

temperatures both immediately and at seven hours after injection.

5

EXAMPLE 15

Effects of human urocortin-related peptide on appetite

The effect of human urocortin-related peptide on appetite was also examined in rats. Cannulas for injection of human urocortin-related peptide were surgically into the right
10 lateral ventricle and the animals were allowed to recover for seven days. During the time, the animals were handled daily to acclimatize the animals to the injection procedure. On the day of injection, the animals were injected with either 5 μ l of saline or 5 μ l of 1 μ g/ μ l human urocortin-related peptide in saline. The
15 amount of food eaten by each animal was recorded every hour for six hours and at fourteen hours.

The total food consumed over the course of the experiments is shown for each time period in Figure 15A. Human urocortin-related peptide injected animals ate significantly less
20 food than control animals. Figure 15B summarizes the amount of food consumed during each time period. hURP treated animals especially ate less food during the first and third hours after injection as well as during the final eight hours of the experiment.

25

EXAMPLE 16Useful urocortin II and human urocortin-related peptide modifications and derivatives

The urocortin II and human urocortin-related peptide disclosed herein most likely represent the prohormone forms of these proteins. It is contemplated that activation of the hormones will involve proteolytic processing and other type of modification to the proteins such as modification resulting in non-amidated forms of the proteins.

Previous studies with ligands for other CRF receptors have shown that a number of amino acid substitutions can be made to these ligands without losing the ability to bind to the receptors of the bioactivity of the ligands. A number of previous studies with urocortin have shown that one, two or even three substitutions are easily tolerated. In some instances modifications to urocortin resulted in protein with more desirable pharmacological properties. Since urocortin II and human urocortin-related peptide are small proteins, such modification can be most easily incorporated by peptide synthesis methods well known to those of skill in the art. These include solid phase techniques, partial solid phase, fragment condensation, and classical solution addition. These methods are especially preferred if nonstandard amino acids are to be incorporated into urocortin II or human urocortin-related peptide. Alternatively, if the modifications consist entirely of natural amino acids, recombinant DNA techniques can be used for mutagenesis and subsequent expression of modified urocortin II and human urocortin-related peptide.

Human urocortin-related peptide lacks a tyrosine residue. Since tyrosine residues are use for the radioiodination of proteins, one possible modification to human urocortin-related peptide would be substitute tyrosine for another amino acid in the protein. Previously, the addition of a sequence consisting of Tyr-Gly to the N-terminal end of urocortin was described. The resulting protein retains CRF receptor binding and bioactivity but would be useful in the radioiodination of the protein. Other N-terminal extensions of the protein of the instant invention may also be constructed for labeling and other purposes.

Deletion of the first seven to ten residues of urocortin was found to result in the formation of effective urocortin antagonists. These proteins were capable of binding to CRF receptors but did not significantly stimulate or activate the receptors. It is expected that deletion of up to five amino acids from urocortin II or human urocortin-related peptide would result in effective antagonists as well. It may also be possible to create antagonists from other urocortin II and human urocortin-related peptide fragments. These antagonists can be effective in elevating levels of the endogenous peptides which are normally cleared by CRF-binding protein. By associating with the CRF-binding protein and blocking CRF, urocortin, urocortin II and human urocortin-related peptide binding to the same protein, the effective *in vivo* concentrations of endogenous CRF, Ucn and Ucn II are increased. Such antagonists can be coadministered with other agonists or antagonists of CRF, Ucn, Ucn II or URP for enhancement of the effects thereof.

Extensive analysis of other CRF receptor binding proteins has shown that substitution of normal amino acids with D-isomer amino acids or cyclizing amino acids results in increased affinity for CRF-receptors. In particular, an especially useful substitution is replacement of the isoleucine residue corresponding to position 9 of SEQ ID No. 3 or SEQ ID No. 11 with a "D-form" isomeric amino acid, preferably D-isoleucine, D-phenylalanine, and D-Leucine. Likewise, a glutamic acid residue corresponding to position 17 of SEQ ID No. 3 or SEQ ID No. 11 can be replaced with D-glutamic acid. Cyclizing amino acids can be formed by chemical bonds between the side chains of two or more residues. For example, adjacent glutamic acid and lysine residues can react to form an amide bond producing a lactam ring. Substitution with nonstandard amino acids such as C α -methylated leucine, C α -methylated alanine, N-im-benzylhistidine, 4-hydroxyproline, 5-hydroxylysine, 3-methylhistidine, homoserine, and ornithine may also be used to form agonists or antagonists of human urocortin-related peptide.

The modifications to urocortin II and human urocortin-related peptide disclosed herein are intended to be illustrative of possible modification that may be performed and are not intended to limit the invention in any way.

Discussion

Genome-wide homology searching was used to identify new members of the CRF family of neuropeptides. One of the new ligands, Ucn II, binds selectively to CRF-R2, is expressed in discrete areas of the rat CNS, and activates central

neurons involved in the processing of visceral sensory information, and in modulating autonomic outflow. Further, Ucn II inhibits food intake, without any effect on gross motor activity.

In addition to a murine peptide that exhibits
5 structural, binding, activity and expression characteristics expected of a CRF family member, a human URP (based on a publicly available EST sequence) was identified which is 80% identical to the mouse sequence at the nucleotide level. However, an important difference evident in the human peptide is the
10 absence of any obvious proteolytic cleavage site that would provide for C-terminal processing of a human homologue. It remains to be determined whether and how any homologous human peptide may be generated from this protein. Nevertheless, whereas Ucn is bound with high affinity by, and
15 signals potently through, both CRF-R1 and CRF-R2 (14-16). Mouse Ucn II and human URP exhibit a high degree of CRF-R2 selectivity in these measures, and will doubtless be of value in dissociating functions mediated by the two receptor types. Figure 16 shows a model of how urocortin II acts on CRF-R1 and CRF-R2.
20 Ucn II binds with high affinity to CRF-R2 and not CRF-R1. Urocortin binds to both receptors while CRF binds with high affinity to CRF-R1 but not to CRF-R2.

Ucn II mRNA displays a limited subcortical distribution in rodent brain that is unique, though ostensibly
25 overlapping in part with those of CRF (paraventricular nucleus; e.g., ref. 31) and Ucn (brainstem and spinal motor nuclei; e.g., ref. 18). Of particular interest is the fact that the transcript is expressed in cell groups involved in stress-related physiologic

and behavioral functions (see ref. 13). This includes the locus
coeruleus, which issues widespread projections to the cortical
mantle and has been implicated in generating levels of arousal
and anxiety (e.g., 32), the paraventricular nucleus, which houses
5 multiple relevant neurosecretory neuron populations and
projects within the CNS to modulate sensory and motor traffic in
central autonomic circuitry (e.g., 33), and the arcuate nucleus,
which has been identified as a pivotal component of an extended
system subserving the regulation of food intake and energy
10 balance (e.g., 34). Although anatomical and functional data to
define the new peptide's place in such contexts are as yet lacking,
the central Ucn II system holds potential for participating in
stress-related functions long implicated as the province of the
broader central CRF network. This contrasts with Ucn, whose
15 dominant seat of cellular expression in brain, the Edinger-
Westphal nucleus, shows very limited capacities in this regard,
largely by virtue of a paucity of documented projections to the
forebrain (16, 18).

In view of its binding characteristics and activity, the
20 failure of the pattern of cellular activation elicited by central Ucn
II to closely mimic the CRF-R2 distribution was unexpected. A
recent study comparing the distribution of Fos expression
induced by icv CRF or UCN documented activation patterns
coarsely consistent with the binding affinities of these peptides
25 for CRF-Rs encoded by the two known genes (35). That is, CRF at
doses similar to those employed here activated sites of CRF-R1
expression in a highly preferential manner, while UCN provoked
Fos induction mainly in subsets of cell groups that express each

receptor. In addition, however, both peptides recruited the very same set of central autonomic structures that were seen here to be the dominant seats of Ucn II-induced activational responses in the rat brain. This is significant in that elements of the central autonomic system are among the best documented sites at which CRF-like peptides can act to elicit stress-related autonomic and behavioral responses. These findings would suggest that type 2, as well as type 1, receptor activation is capable of engaging this system, though the basis for this is unclear. Among the nodal points in the central autonomic network, only the parabrachial nucleus (R1) and the NTS (R2) have been identified as sites of CRF-R expression (27, 28, 35) and it remains to be determined whether receptor-mediated activation of either or both of these is sufficient to enlist the system as a whole. It is important to note that systemic injections of synthetic Ucn II failed to elicit comparably powerful activational responses within central autonomic cell groups over the same range of doses that were used for icv injection studies. This is an important control, as activation of peripheral CRF-R2 can yield a marked and persistent reduction in blood pressure (16, 17), and salient hypotensive challenges are capable of activating the very same central autonomic structures as are responsive to central Ucn II administration (36, 37).

The initial characterization of the effects of icv Ucn II on food intake and activity complements recent efforts to tease apart the roles of individual CRF-Rs in stress-related behaviors. For example, while mice bearing null mutations of either receptor display normal basal food intake, CRF-R1-deficient animals have

been shown to be refractory to the anorexic effects of UCN during the period immediately following injection, but not at later time points, while the converse is true of CRF-R2 mutant mice (11, 38, 39). This has been taken as suggesting that the early and later
5 phase of Ucn-mediated feeding suppression may be CRF-R1- and CRF-R2-mediated events, respectively. Using a different paradigm (nighttime free-feeding rather than deprivation-induced refeeding) provided data supportive of such a parsing, as the R2-specific ligand did not reliably suppress food intake at the
10 early time points, but did so beyond 6 hrs post-injection.

Measures of motor activity also supported a dissociation of CRF-R involvement in this parameter. In line with recent evidence in knockout mice suggesting locomotor activation to be a CRF-R1-mediated event (40), it was found that the R1-
15 selective agonist, CRF, significantly increased gross motor activity, while UCN II administration did not. Interestingly, treatment with UCN, which is bound with high affinity by both receptors, resulted in a nonsignificant trend toward increased activity, with values being reliably lower than those seen in
20 response to CRF. This is coarsely consistent with a growing body of evidence to support a functional antagonism between the two known receptor types. Whereas CRF-R1-deficient mice show reduced endocrine and anxiety-like responses to stress (41), CRF-R2 mutant lines display increases in these parameters (11, 39,
25 42) suggesting that basal activation of CRF-R2 may play a role in opposing CRF-R1-driven stress responses.

The identification of an endogenous CRF-R2-selective ligand will allow for more detailed analysis of the roles of

individual CRF-related signaling molecules in stress-related physiologic and behavioral functions. Central expression of Ucn II mRNA identified cell groups that respond to central administration of the peptide, and confirmed behavioral
5 responses that are consistent with previously hypothesized consequences of CRF-R2 activation. Further insight into the place of this peptide in stress biology will require delineation of the central projections of Ucn II containing cells, and identification of the factors and circumstances that regulate gene expression and
10 peptide release.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each
5 individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those
10 inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses
15 will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. DNA encoding urocortin II selected from the group consisting of:

5 (a) isolated and purified DNA which encodes urocortin II protein;

(b) isolated and purified DNA which hybridizes at high stringency conditions to the antisense complement of the isolated DNA of (a) above, wherein high stringency conditions are
10 characterized as membrane washing at high temperature and low salt concentration functionally equivalent to 0.1 x SSC at 65°C, wherein said DNA encodes urocortin II protein; and

(c) isolated and purified DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to the
15 degeneracy of the genetic code, and which encodes urocortin II protein.

2. The DNA of claim 1, wherein said DNA encodes an urocortin II protein precursor peptide of amino acid sequence
20 SEQ ID No: 10.

3. The DNA of claim 1, wherein said DNA encodes a urocortin II protein, wherein said protein has a amino acid sequence of SEQ ID No. 11.

25 4. A vector capable of expressing the DNA of claim 1 wherein said vector comprises said DNA and regulatory elements necessary for expression of said DNA in a cell.

5. The vector of claim 4, wherein said DNA encodes a urocortin II protein having the amino acid sequence shown in SEQ ID No: 11.

5 6. A host cell transfected with the vector of claim 4, said vector expressing urocortin II protein.

7. The host cell of claim 6, wherein said cell is selected from group consisting of bacterial cells, mammalian
10 cells, plant cells and insect cells.

8. The host cell of claim 7, wherein said bacterial cell is *E. coli*.

15 9. Isolated and purified urocortin II protein coded for by DNA selected from the group consisting of:

(a) isolated and purified DNA which encodes urocortin II protein;

(b) isolated and purified DNA which hybridizes at
20 high stringency conditions to the antisense complement of the isolated DNA of (a) above, wherein high stringency conditions are characterized as membrane washing at high temperature and low salt concentration functionally equivalent to 0.1 x SSC at 65°C, wherein said DNA encodes urocortin II protein; and

25 (c) isolated and purified DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to the degeneracy of the genetic code, and which encodes urocortin II protein.

10. The isolated and urocortin II protein of claim 9 having the amino acid sequence shown in SEQ ID No: 11.

11. An antibody directed against the urocortin II
5 protein of claim 9.

12. The antibody of claim 11, wherein said antibody is a monoclonal antibody.

10 13. A pharmaceutical composition comprising the urocortin II protein of claim 9 and a pharmaceutically acceptable carrier.

15 14. A method of treating a pathophysiological state, comprising the step of administering the pharmaceutical composition of claim 13 to an individual in need of such treatment.

20 15. The method of claim 14, wherein said pathophysiological state is selected from the group consisting of high body temperature, appetite dysfunction, congestive heart failure, stress, anxiety, and undesirably low levels of ACTH secretion.

25 16. DNA encoding human urocortin-related peptide selected from the group consisting of:

(a) isolated and purified DNA which encodes a human urocortin-related peptide protein;

(b) isolated and purified DNA which hybridizes at high stringency conditions to the antisense complement of the isolated DNA of (a) above, wherein high stringency conditions are characterized as membrane washing at high temperature and low salt concentration functionally equivalent to 0.1 x SSC at 65°C, wherein said DNA encodes a human urocortin-related peptide protein; and

(c) isolated and purified DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to the degeneracy of the genetic code, and which encodes a human urocortin-related peptide protein.

17. The DNA of claim 16, wherein said DNA has the sequence shown in SEQ ID No:1.

18. The DNA of claim 16, wherein said DNA encodes a human urocortin-related peptide precursor protein of amino acid sequence SEQ ID No: 2.

19. The DNA of claim 16, wherein said DNA encodes a human urocortin-related peptide protein, wherein said protein has a amino acid sequence of SEQ ID No. 3.

20. A vector capable of expressing the DNA of claim 16 wherein said vector comprises said DNA and regulatory elements necessary for expression of said DNA in a cell.

21. The vector of claim 20, wherein said DNA encodes a human urocortin-related peptide protein having the amino acid sequence shown in SEQ ID No: 3.

5 22. A host cell transfected with the vector of claim 20, said vector expressing a human urocortin-related peptide protein.

23. The host cell of claim 22, wherein said cell is
10 selected from group consisting of bacterial cells, mammalian cells, plant cells and insect cells.

24. The host cell of claim 23, wherein said bacterial cell is *E. coli*.

15 25. Isolated and purified human urocortin-related peptide coded for by DNA selected from the group consisting of:

(a) isolated and purified DNA which encodes a human urocortin-related peptide protein;

20 (b) isolated and purified DNA which hybridizes at high stringency conditions to the antisense complement of the isolated DNA of (a) above, wherein high stringency conditions are characterized as membrane washing at high temperature and low salt concentration functionally equivalent to 0.1 x SSC at 65°C,
25 wherein said DNA encodes a human urocortin-related peptide protein; and

(c) isolated and purified DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to the

degeneracy of the genetic code, and which encodes a human urocortin-related peptide protein.

26. The isolated and purified human urocortin-
5 related peptide protein of claim 25 having the amino acid sequence shown in SEQ ID No: 3.

27. An antibody directed against the human urocortin-related peptide protein of claim 25.

10

28. The antibody of claim 27, wherein said antibody is a monoclonal antibody.

29. A pharmaceutical composition comprising the
15 human urocortin-related peptide protein of claim 25 and a pharmaceutically acceptable carrier.

30. A method of treating a pathophysiological state, comprising the step of administering the pharmaceutical
20 composition of claim 29 to an individual in need of such treatment.

31. The method of claim 30, wherein said pathophysiological state is selected from the group consisting of
25 high body temperature, appetite dysfunction, congestive heart failure, stress, anxiety, and undesirably low levels of ACTH secretion.

32. A modified protein, wherein said protein is selected from the group consisting of urocortin II and human urocortin-related peptide.

5 33. The protein of claim 32, wherein said protein has been mutated to contain a tyrosine residue.

 34. The protein of claim 32, wherein said protein has been modified by an addition of a sequence consisting of Tyr-
10 Gly to the N-terminal end of said protein

 35. The protein of claim 32, wherein said protein has been modified by an N-terminal deletion, wherein said deletion comprises amino acids selected from the group
15 consisting of the first amino acid, the first and second amino acids, the first through third amino acids, the first through fourth amino acids, and the first through fifth amino acids.

 36. The protein of claim 32, wherein said protein is
20 selected from the group consisting of proteins of sequence SEQ ID No. 3 and SEQ ID No. 11 and wherein an isoleucine residue corresponding to position 9 of said is replaced with a "D-form" isomeric amino acid.

 37. The protein of claim 36, wherein said "D-form"
25 isomeric amino acid is selected from the group consisting of D-isoleucine, D-phenylalanine, and D-leucine.

38. The protein of claim 32, wherein said protein is selected from the group consisting of urocortin II of sequence SEQ ID No. 11 and human urocortin-related protein of sequence SEQ ID No. 11, wherein a glutamic acid residue corresponding to
5 position 17 of said protein is replaced with D-glutamic acid.

39. The protein of claim 32, wherein amino acids are replaced with nonstandard amino acids known in the art.

10 40. The protein of claim 39, wherein said nonstandard amino acid are selected from the group consisting of C_α-methylated leucine, C_α-methylated alanine, N-im-benzylhistidine, 4-hydroxyproline, 5-hydroxylysine, 3-methylhistidine, homoserine, and ornithine.

15 41. The protein of claim 32, wherein said protein has been acylated at the N-terminus of said protein.

42. The protein of claim 32, wherein said protein is acylated with a fatty acid.

20

43. The protein of claim 32, wherein said protein has been modified to contain a fluorescent label.

44. A conjugate of the protein of claim 32 linked to
25 a toxin.

45. A conjugate of the protein of claim 32 with a complexing agent for radionuclides.

46. The conjugate of claim 45 complexed with a
5 radionuclide.

CTC ACG ATG ACC AGG TGT GCT CTG CTG TTG CTG ATG GTC GGC AGA GTC CTG
 M T R C A L L L L M V L M L G R V L
 10
 GTT GTC CCA GTG ACC CCT ATC CCA ACC TTC CAG CTC CGC CCT CAG AAT TCT CCC CAG ACC
 V P V T P I P T F Q L R P Q N S P Q T
 20
 ACT CCC CGA CCT GCG GCC TCA GAG AGC CCC TCA GCT GCT CCC ACA TGG CCG TGG GCT GCC
 T P R P A A S E S P S A A P T W P W A A
 30
 CAG AGC CAC TGC AGC CCC ACC CGC CAC CCT GGC TCG CGC ATT GTC CTA TCG CTG GAT GTC
 Q S H C S P T R H P G S R I V L S L D V
 40
 CCC ATC GGC CTC TTG CAG ATC TTA CTG GAG CAA GCC CGG GCC AGG GCT GCC AGG AGG CAG
 P I G L L Q I L L L E Q A R A R A R E Q
 50
 GCC ACC AAC GCC CGC ATC CTG GCC CGT GTC GGC CAC TGC TGA GCC TGA GAG AGG GGG
 A T T N A R I L A R V G H C * (SEQ ID NO:2)
 60
 TCA CAG TGA TAG GGC CAC CCT GGA TGG GAA GAC CTG GAG (SEQ ID NO:1)
 70
 80
 90
 100
 110

Fig. 1

<p>CTC ACG ATG ACC AGG TGT GCT CTG CTG TTG CTG ATG GTC CTG ATG TTG GGC AGA GTC CTG</p> <p>M T R C A L L L L L M V L M L G R V L</p>	10
<p>GTT GTC CCA GTG ACC CCT ATC CCA ACC TTC CAG CTC CGC CCT CAG AAT TCT CCC CAG ACC</p> <p>V P V T P I P T F Q L R P Q N S P Q T</p>	30
<p>ACT CCC CGA CCT GCG GCC TCA GAG AGC CCC TCA GCT GCT CCC ACA TGG CCG TGG GCT GCC</p> <p>T P R P A A S E S P S A A P T W P W A A</p>	50
<p>CAG AGC CAC TGC AGC CCC ACC CGC CAC CCT GGC TCG CGC ATT GTC CTA TCG CTG GAT GTC</p> <p>Q S H C S P T R H P G S R I V L S L D V</p>	70
<p>CCC ATC GGC CTC TTG CAG ATC TTA CTG GAG CAA GCC CGC GGC AGG GCT GCC AGG GAG CAG</p> <p>P I G L L Q I L L E Q A A R A A R E Q</p>	90
<p>GCC ACC ACC AAC GCC CGC ATC CTG GCC CGT GTC GGC CAC TGC TGA GCC TGA GAG AGG GGG</p> <p>A T T N A R I L A R V G H C * (SEQ ID NO:2)</p>	110
<p>TCA CAG TGA TAG GGC CAC CCT GGA TGG GAA GAC CTG GAG (SEQ ID NO:1)</p>	

Fig. 2

hURP	IVISIDVPIGFIQILLTEGARARARARHGAHINAFIARV	38/38	(SEQ ID NO: 4)
hUcn	DNPSISIDLTFFHRTTTELARTOSQRRRAEQNRITFDNV	16/38	(SEQ ID NO: 5)
hCRF	SEEPPISEDLTFHRTREVLEMARAEQLAQAHSNRKLMEII	13/38	(SEQ ID NO: 6)
cUro	NDDPPISIDLTFFHRTNMIEMARNENQRECAFNRKYHDEV	14/38	(SEQ ID NO: 7)
fsvg	-EGPPISIDLSLETRKMIHIEKQEKQGAANRLLHDTI	10/38	(SEQ ID NO: 8)
dCRF/Uro	PAETPNSSDLTFHRTREMIEIAKHENQQMGAADSNREIMDTI	12/38	(SEQ ID NO: 9)

Fig. 3

MTRWALVVFVVLMLDRILEFVPGTPIPTFQLLPQNSLETP 40
SSVTSESSSGTTGPSASWSNSKASPYPDTRVILSLDVPI 80
GLLRILLEQARYKAARNQAATNAQILAHVGR (SEQ ID NO: 10) 112

Fig. 4A

	mouse Ucn II:	•	(SEQ ID NO: 10)
	human URP:	•	(SEQ ID NO: 11)
	fish URP:	•	(SEQ ID NO: 12)
	rat Ucn:	•	(SEQ ID NO: 13)
	r/hCRF:..	•	(SEQ ID NO: 14)

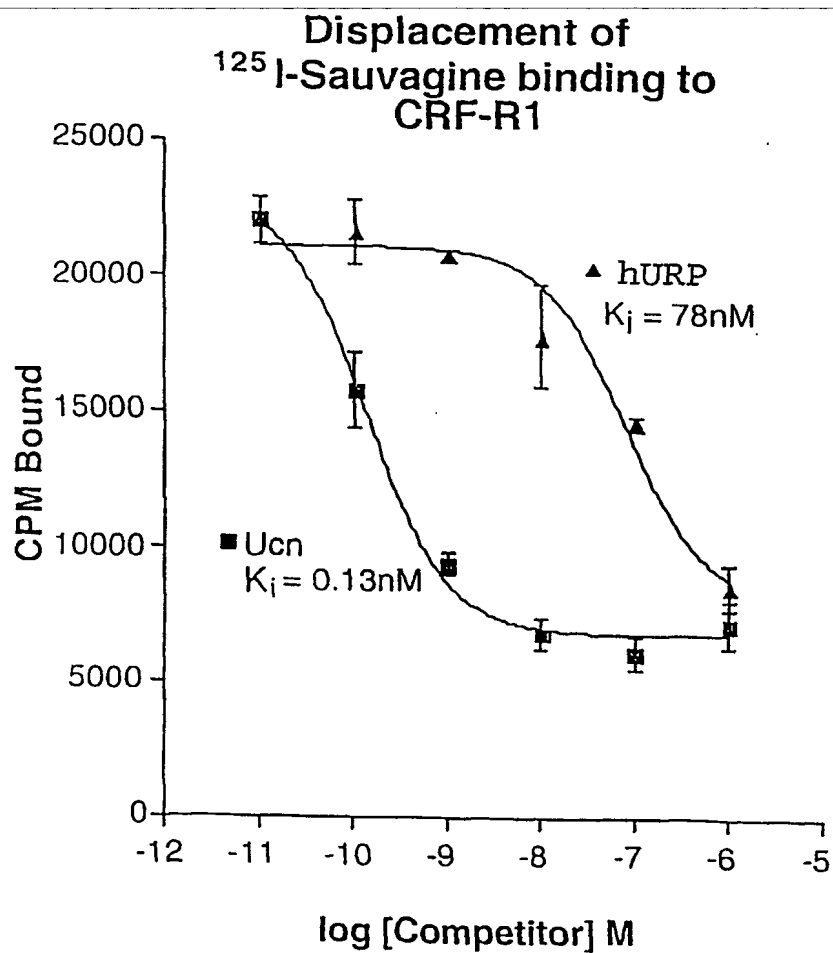


Fig. 5A

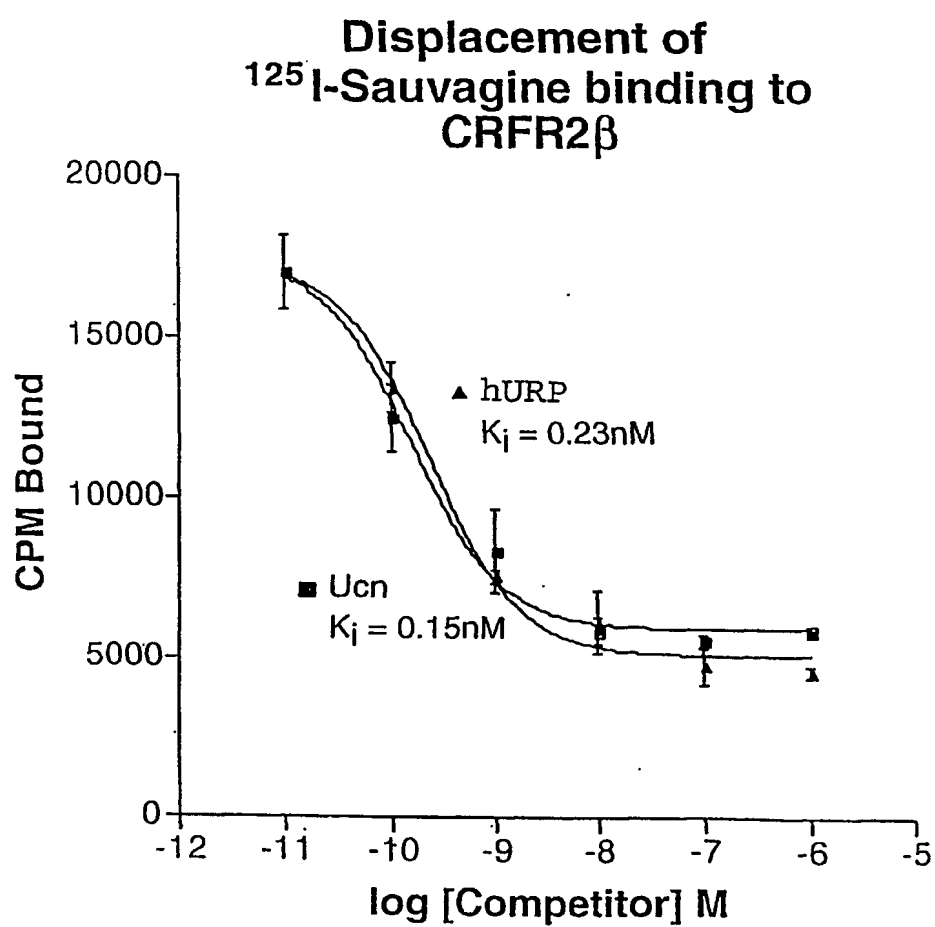


Fig. 5B

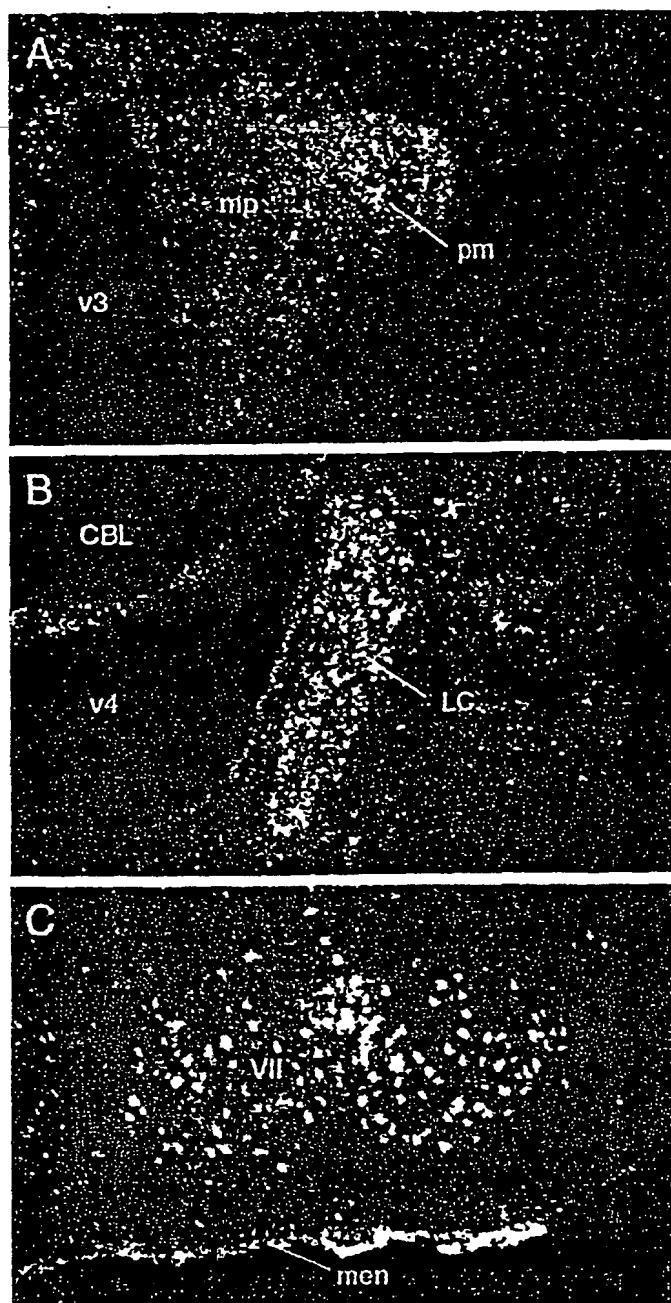


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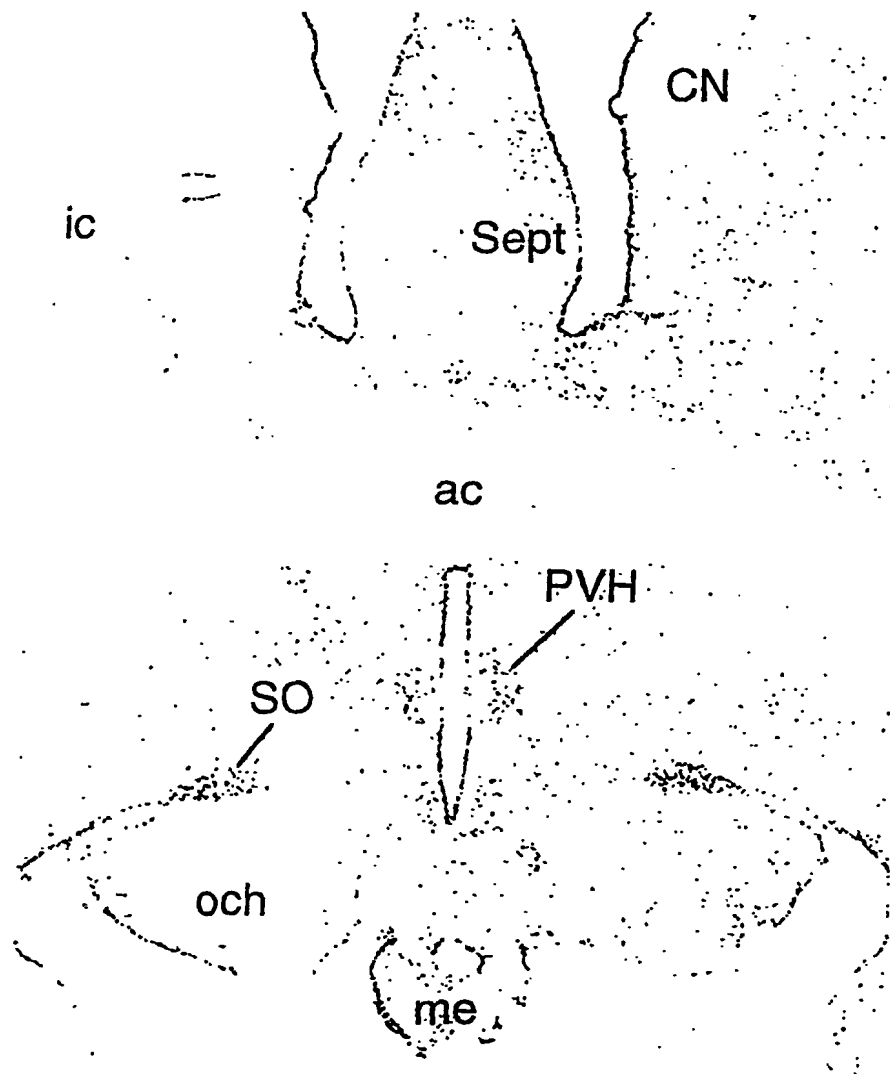


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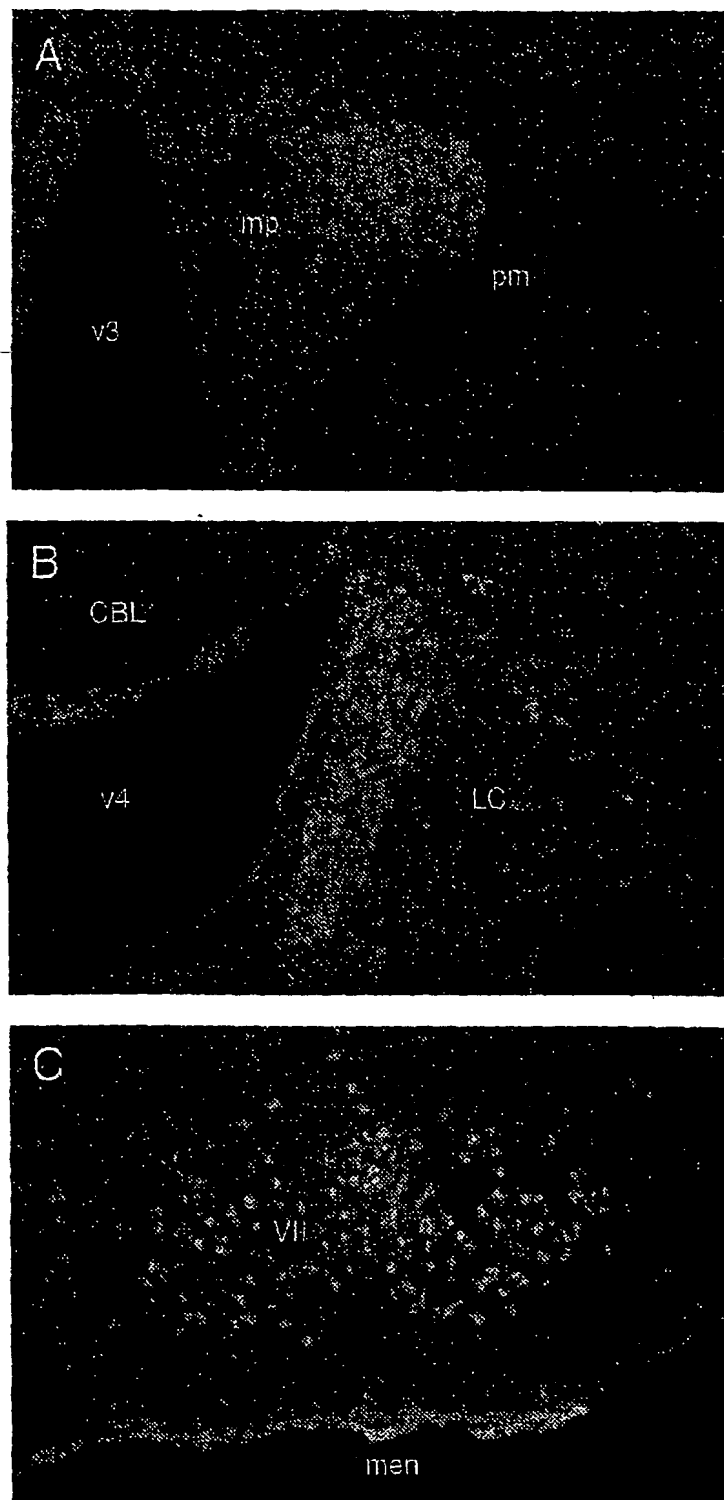


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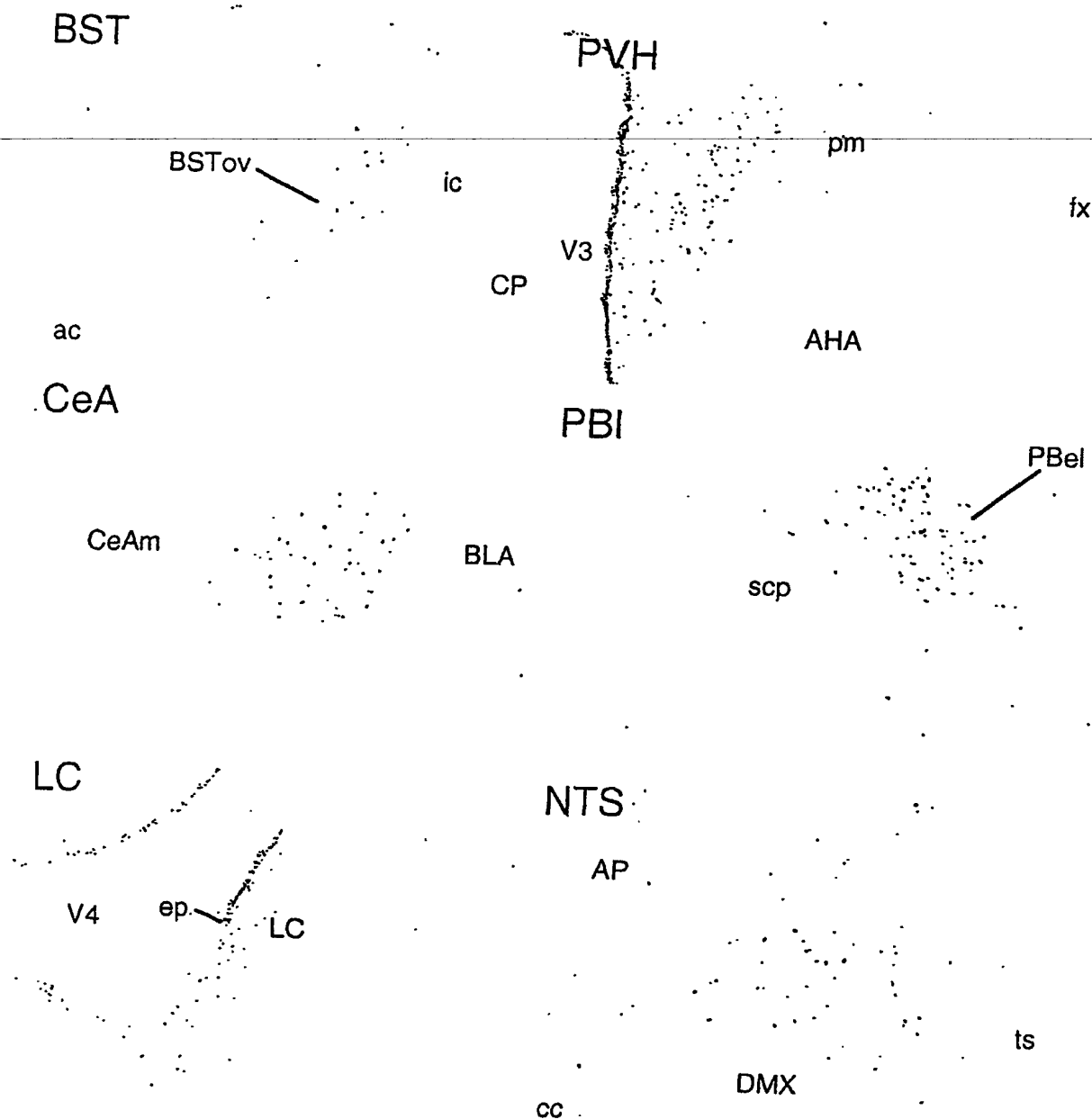


Fig. 9

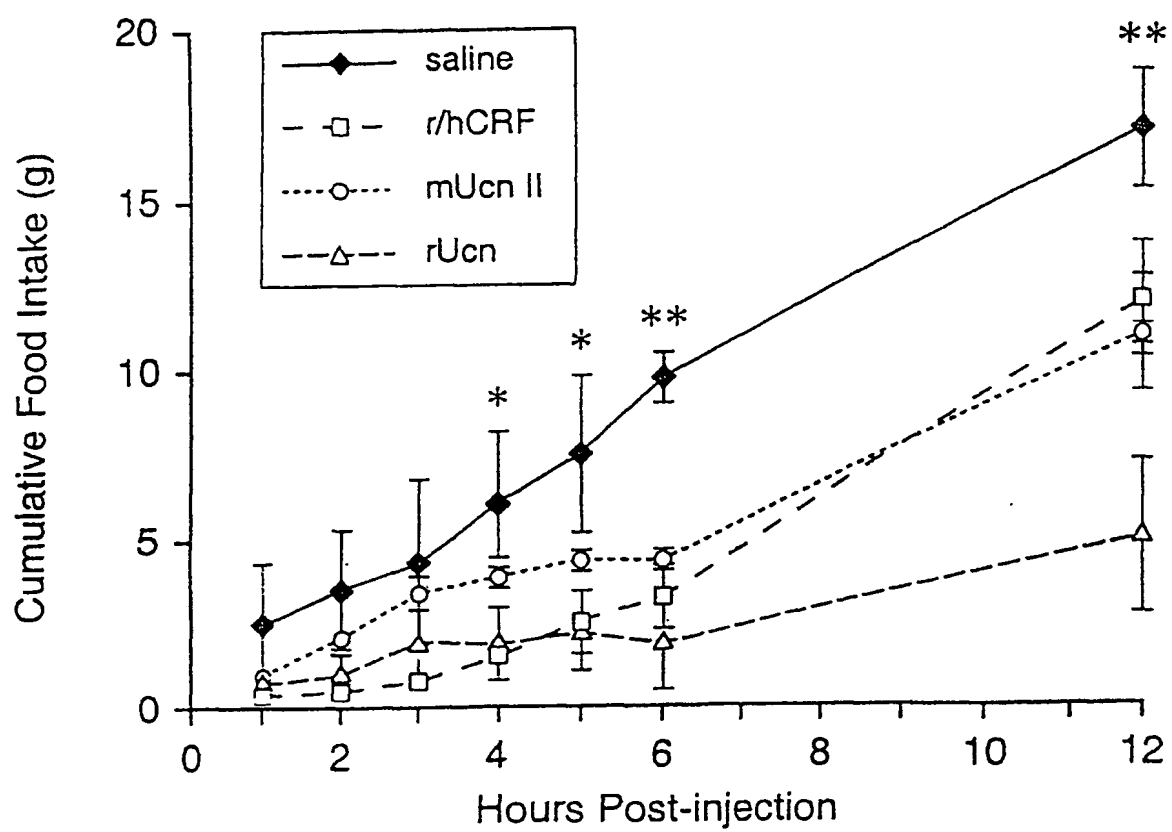


Fig. 10A

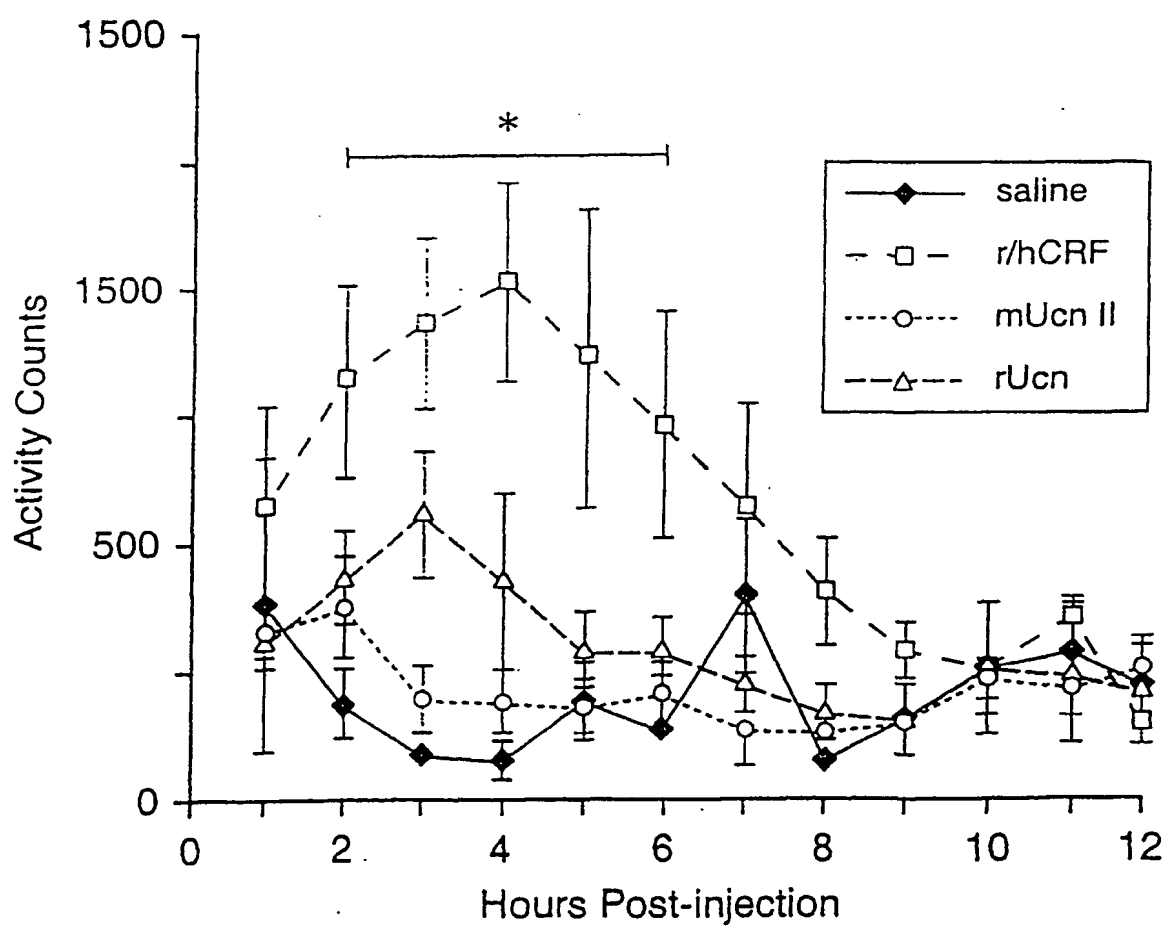


Fig. 10B

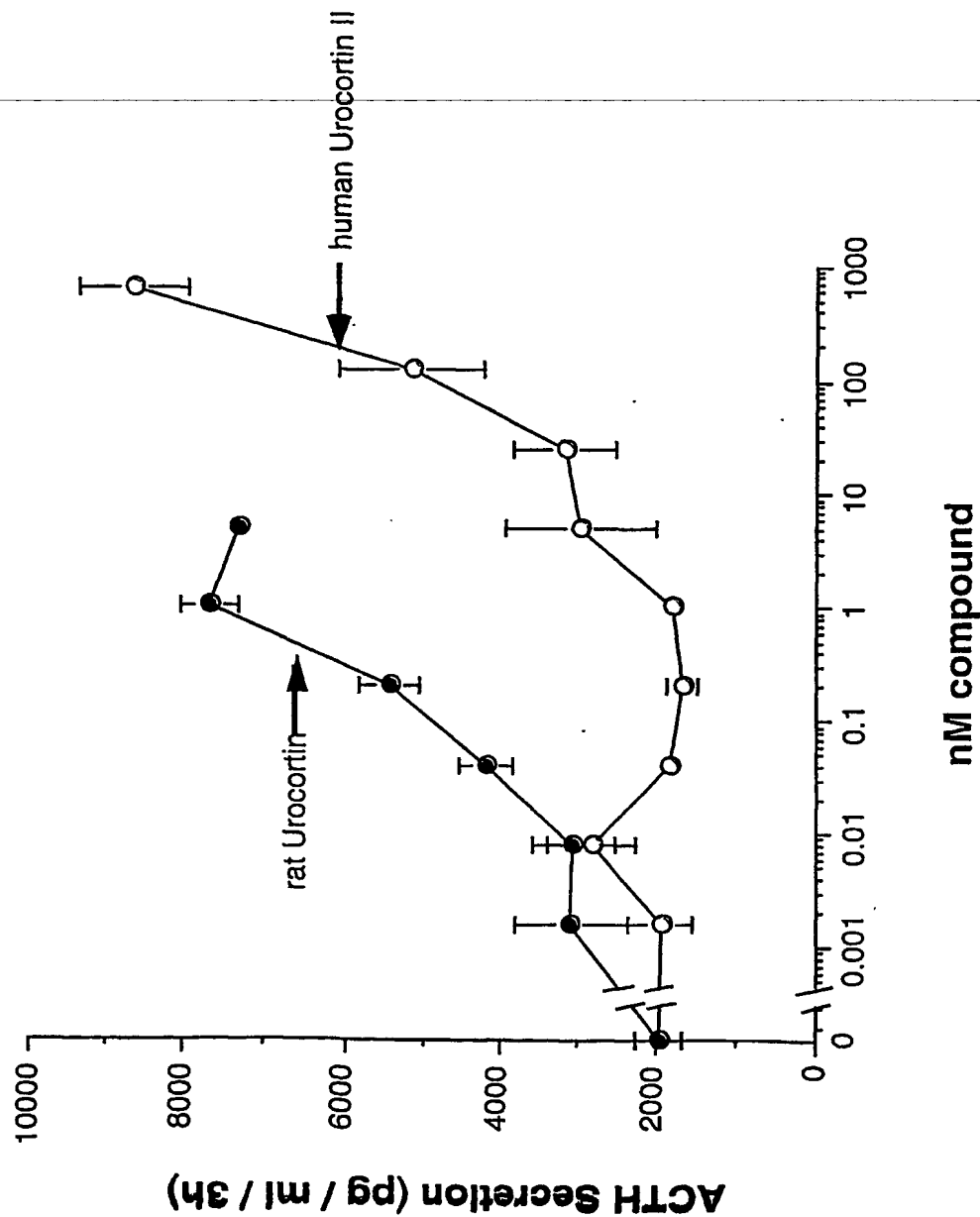


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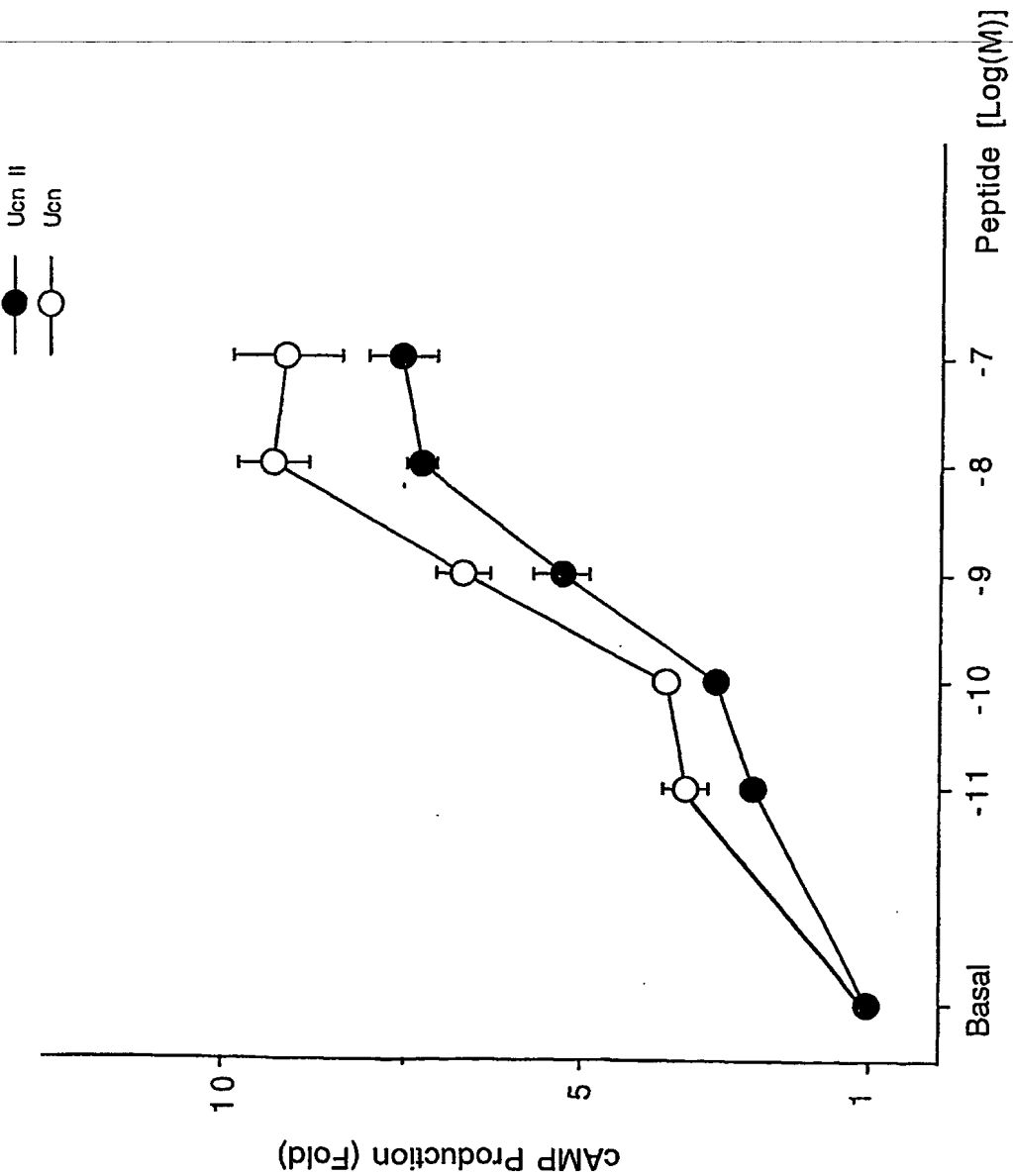
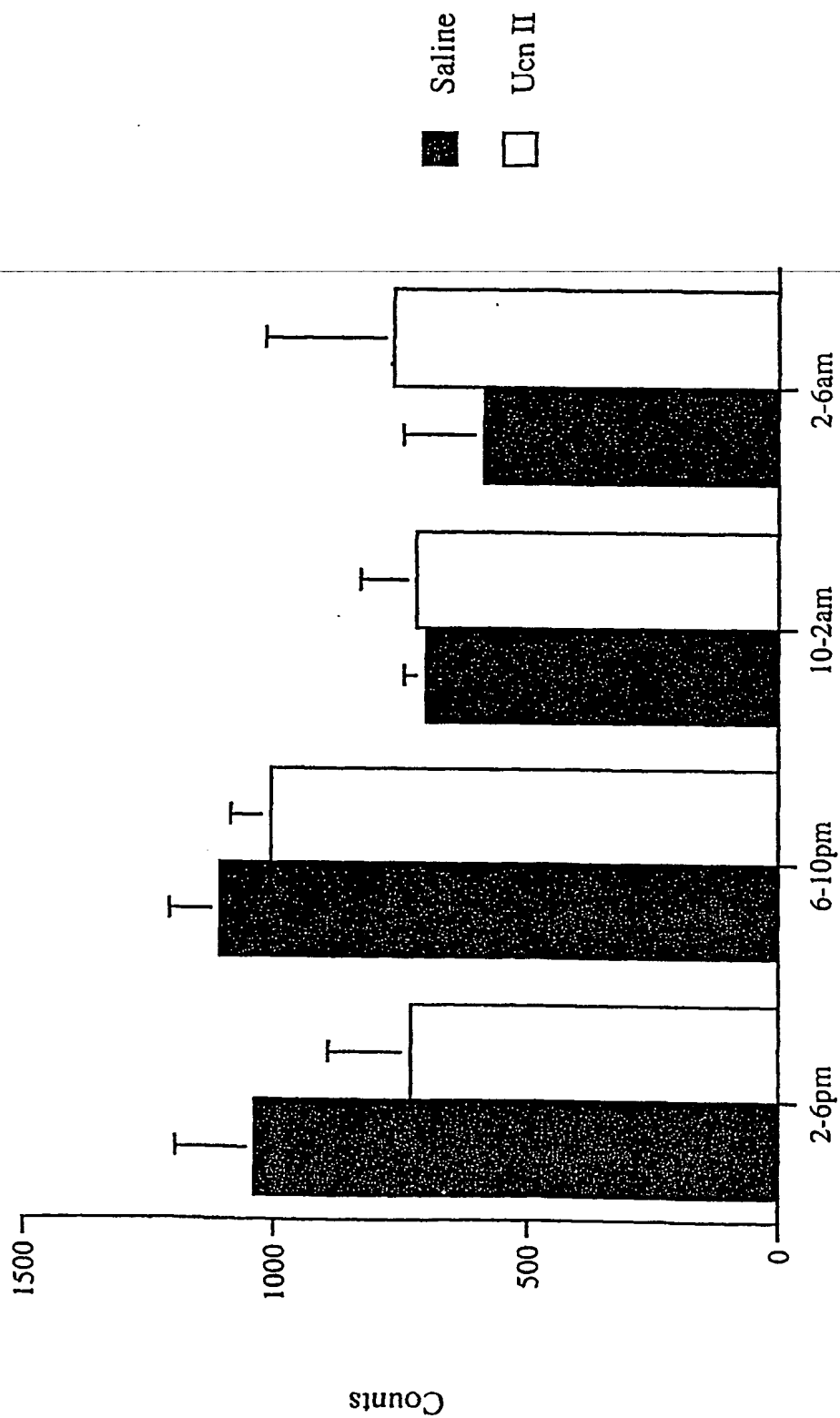
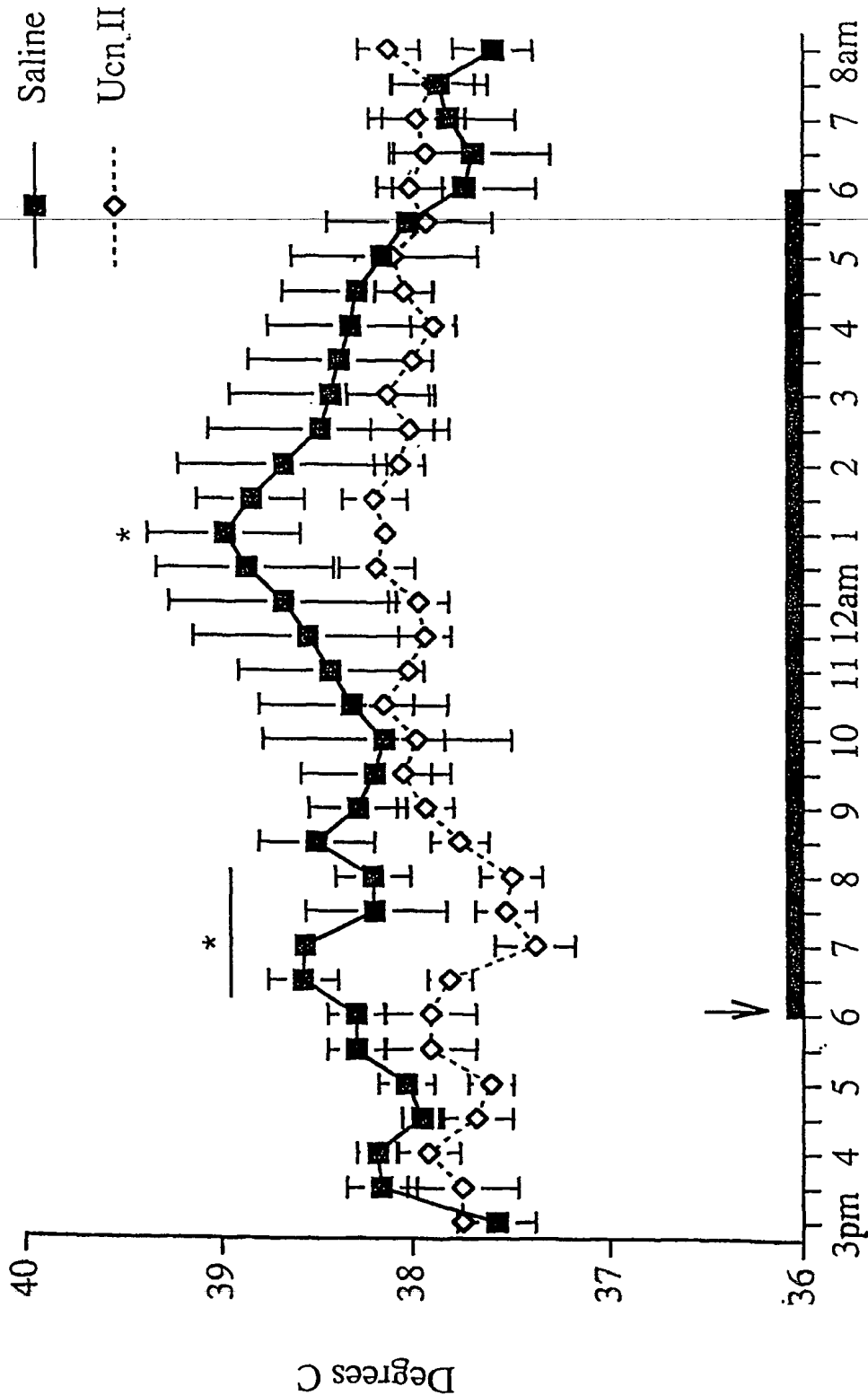


Fig. 12



Time of Day

Fig. 13



Time of Day

Fig. 14

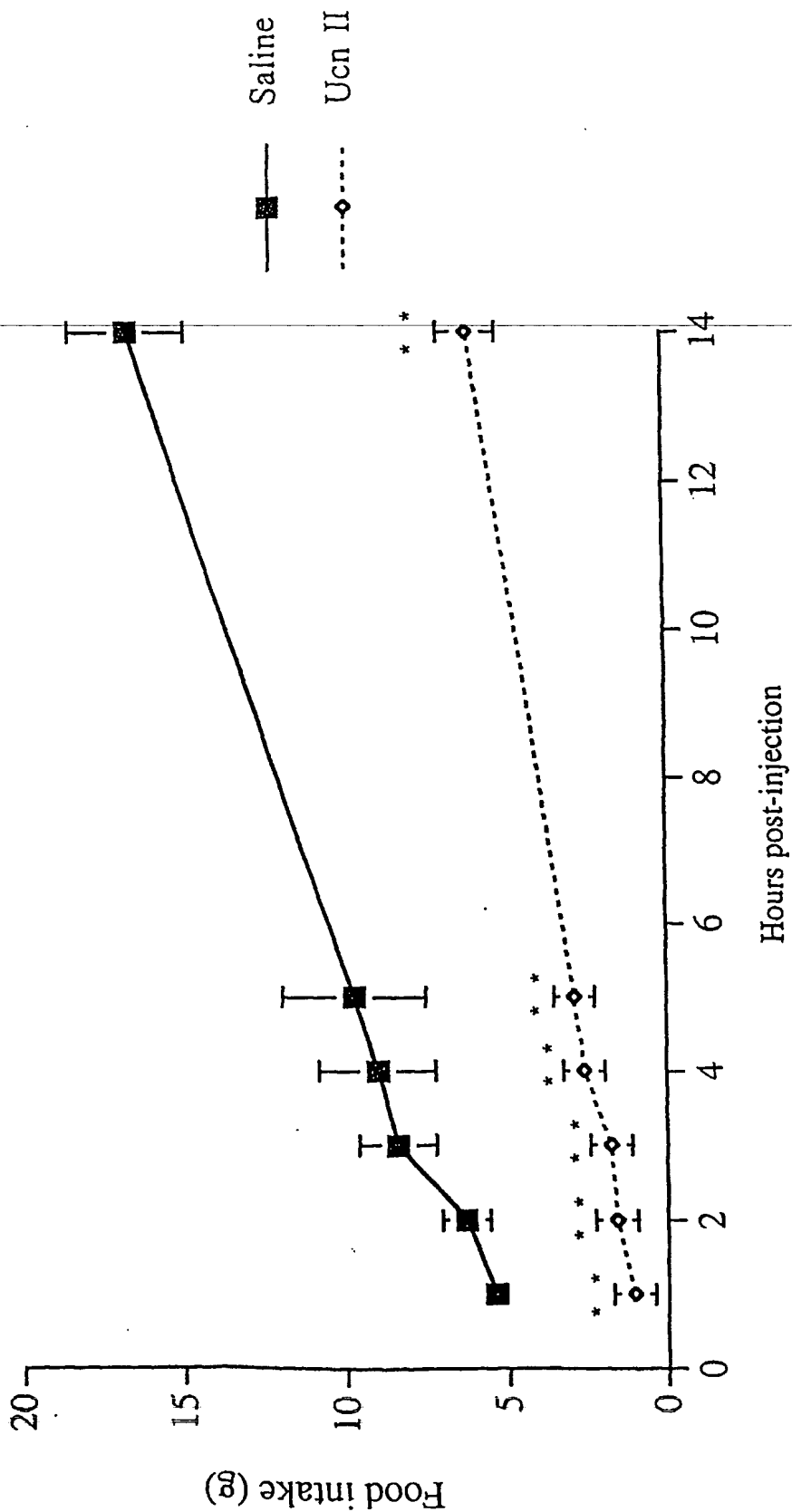


Fig. 15A

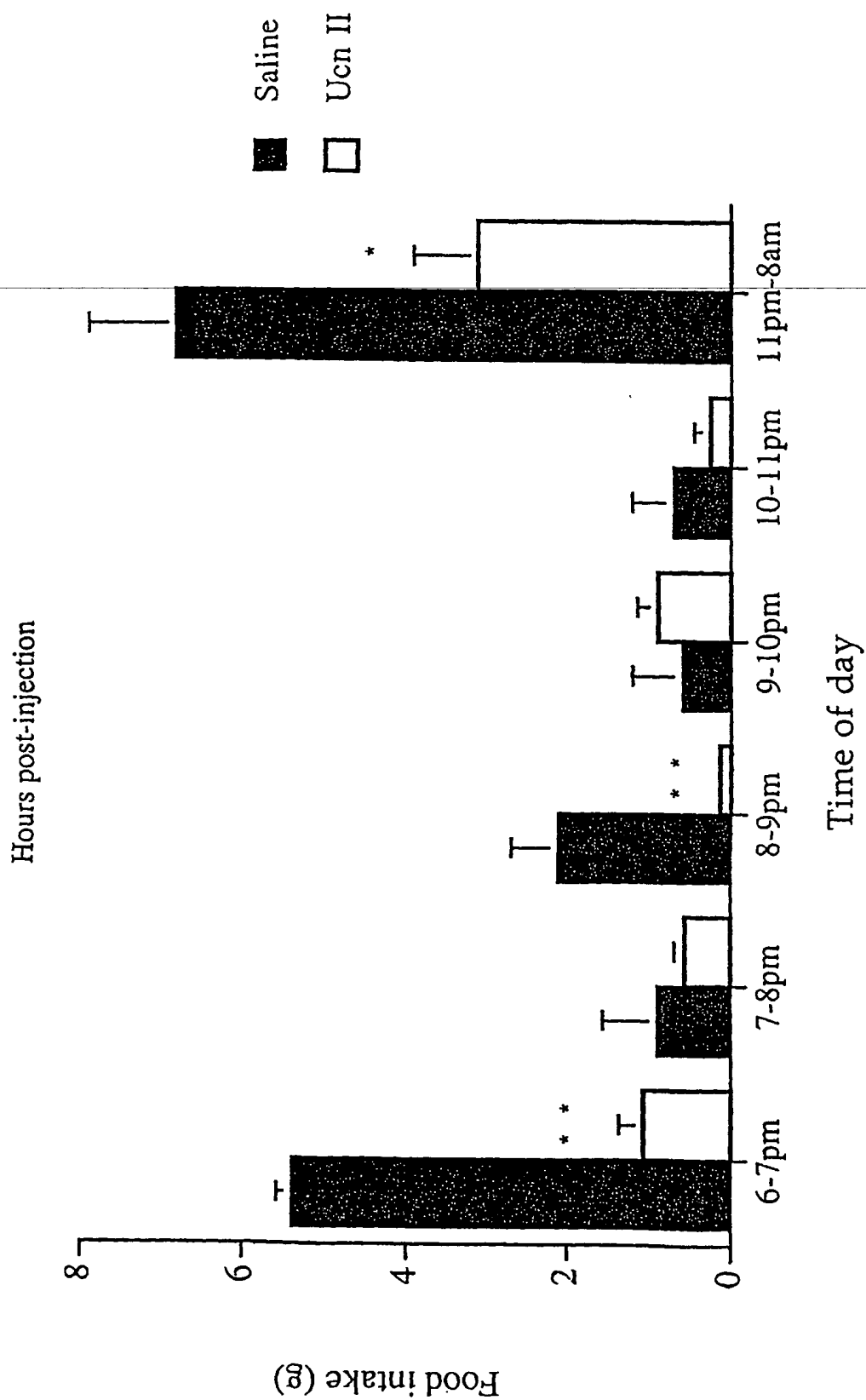


Fig. 15B

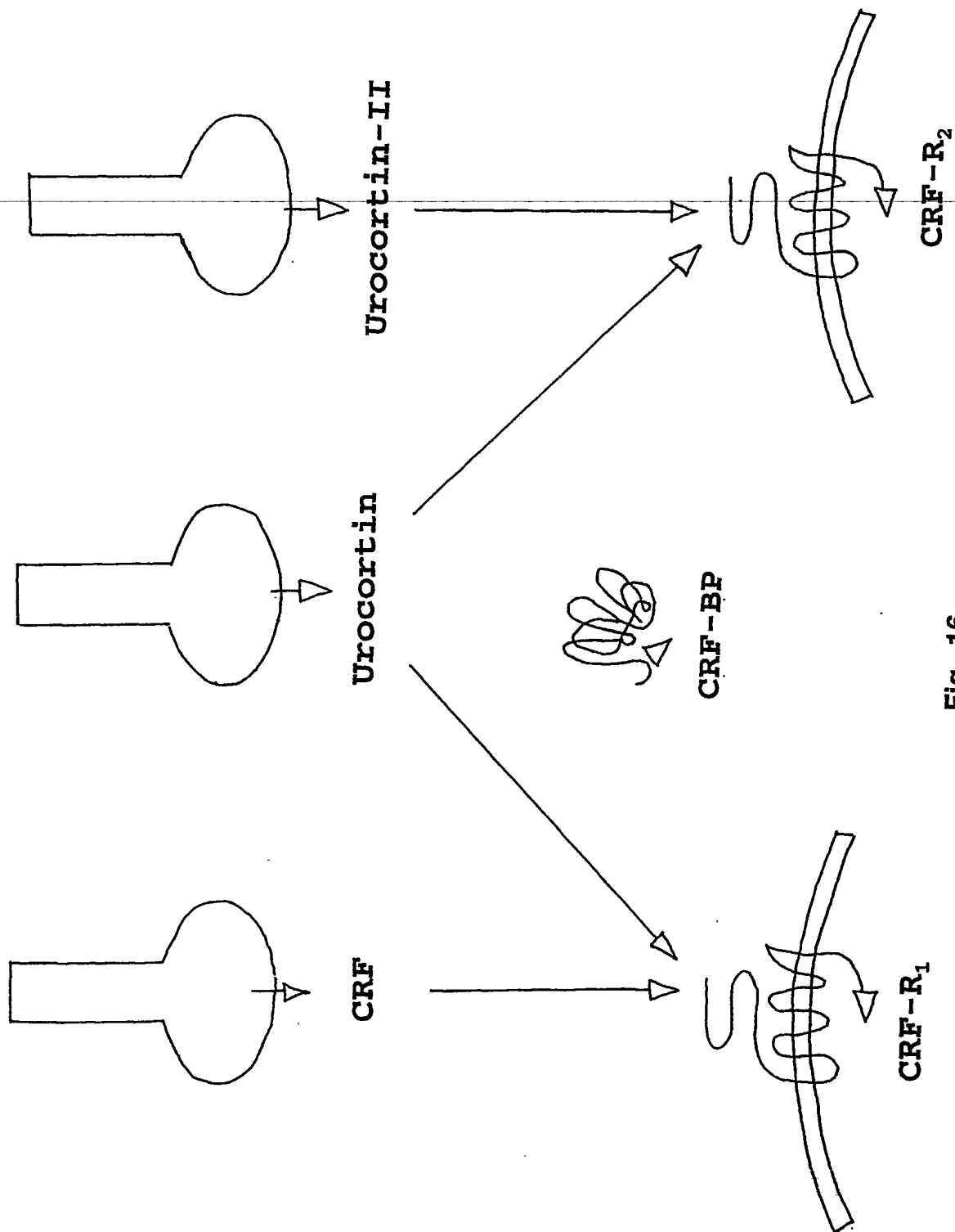


Fig. 16

SEQUENCE LISTING

<110> Vale, Wylie Walker Jr.
Lewis, Kathy Ann
Reyes, Teresa Marie
Hogenesch, John Beren
Sawchenko, Paul Emil
Vaughan, Joan Maureen
Rivier, Jean Edouard Frederic
Perrin, Marilyn Heller

<120> Urocortin Proteins and Uses Thereof

<130> D6334PCT

<141> 2001-08-04

<150> US 60/273,969

<151> 2001-03-07

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ctcagaattc tccccagacc actccccgac ctgcggcctc agagagcccc 150
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<210> 2

<211> 112

<212> PRT

<213> *Homo sapiens*

<220>

<223> Human Urocortin-related peptide (hURP)
 precursor peptide

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Arg	Val	Leu	Val	Val	Pro	Val	Thr	Pro	Ile	Pro	Thr	Phe	Gln	Leu	20	25	30
Arg	Pro	Gln	Asn	Ser	Pro	Gln	Thr	Thr	Pro	Arg	Pro	Ala	Ala	Ser	35	40	45
Glu	Ser	Pro	Ser	Ala	Ala	Pro	Thr	Trp	Pro	Trp	Ala	Ala	Gln	Ser	50	55	60
His	Cys	Ser	Pro	Thr	Arg	His	Pro	Gly	Ser	Arg	Ile	Val	Leu	Ser	65	70	75
Leu	Asp	Val	Pro	Ile	Gly	Leu	Leu	Gln	Ile	Leu	Leu	Glu	Gln	Ala	80	85	90
Arg	Ala	Arg	Ala	Ala	Arg	Glu	Gln	Ala	Thr	Thr	Asn	Ala	Arg	Ile	95	100	105
Leu	Ala	Arg	Val	Gly	His	Cys									110		

<210> 3

<211> 41

<212> PRT

<213> *Homo sapiens*

<220>

<223> Human Urocortin-related peptide (hURP)

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Ile Val Leu Ser Leu Asp Val Pro Ile Gly Leu Leu Gln Ile Leu
 5 10 15

Leu Glu Gln Ala Arg Ala Arg Ala Ala Arg Glu Gln Ala Thr Thr
 20 25 30

Asn Ala Arg Ile Leu Ala Arg Val Gly His Cys
 35 40

<210> 4

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<213> *Homo sapiens*

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<223> Amino acids 1-38 of human
 Urocortin-related peptide (hURP)

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 5 10 15

Leu Glu Gln Ala Arg Ala Arg Ala Ala Arg Glu Gln Ala Thr Thr
 20 25 30

Asn Ala Arg Ile Leu Ala Arg Val
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<210> 5

<211> 40

<212> PRT

<213> *Homo sapiens*

<220>

<223> Human Urocortin (hUcn)

<400> 5

Asp Asn Pro Ser Leu Ser Ile Asp Leu Thr Phe His Leu Leu Arg
 5 10 15

Thr Leu Leu Glu Leu Ala Arg Thr Gln Ser Gln Arg Glu Arg Ala
 20 25 30

Glu Gln Asn Arg Ile Ile Phe Asp Ser Val
35 40

<210> 6

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<212> PRT

<213> *Homo sapiens*

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<223> Human Corticotropin Releasing Factor

<400> 6

Ser Glu Glu Pro Pro Ile Ser Leu Asp Leu Thr Phe His Leu Leu
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Arg Glu Val Leu Glu Met Ala Arg Ala Glu Gln Leu Ala Gln Gln
20 25 30

Ala His Ser Asn Arg Lys Leu Met Glu Ile Ile
35 40

<210> 7

<211> 41

<212> PRT

<213> *Cyprinus carpio*

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<223> Carp Urotensin (cUro)

<400> 7

Asn Asp Asp Pro Pro Ile Ser Ile Asp Leu Thr Phe His Leu Leu
5 10 15

Arg Asn Met Ile Glu Met Ala Arg Asn Glu Asn Gln Arg Glu Gln
20 25 30

Ala Gly Leu Asn Arg Lys Tyr Leu Asp Glu Val
35 40

<210> 8

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<212> PRT

<213> Unknown

<220>

<223> Frog Sauvagine (fSvg)

<400> 8

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Lys	Met	Ile	Glu	Ile	Glu	Lys	Gln	Glu	Lys	Glu	Lys	Gln	Gln	Ala
				20					25					30

Ala	Asn	Asn	Arg	Leu	Leu	Leu	Asp	Thr	Ile
				35					40

<210> 9

<211> 41

<212> PRT

<213> Unknown

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(dCRF/Uro)

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				5					10					15

Arg	Glu	Met	Ile	Glu	Ile	Ala	Lys	His	Glu	Asn	Gln	Gln	Met	Gln
				20					25					30

Ala	Asp	Ser	Asn	Arg	Arg	Ile	Met	Asp	Thr	Ile
				35					40	

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<212> PRT

<213> *Mus musculus*

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<223> Mouse Urocortin II precursor peptide

<400> 10

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				5					10					15

Arg Ile Leu Phe Val Pro Gly Thr Pro Ile Pro Thr Phe Gln Leu
 20 25 30
 Leu Pro Gln Asn Ser Leu Glu Thr Thr Pro Ser Ser Val Thr Ser
 35 40 45
 Glu Ser Ser Ser Gly Thr Thr Thr Gly Pro Ser Ala Ser Trp Ser
 50 55 60
 Asn Ser Lys Ala Ser Pro Tyr Leu Asp Thr Arg Val Ile Leu Ser
 65 70 75
 Leu Asp Val Pro Ile Gly Leu Leu Arg Ile Leu Leu Glu Gln Ala
 80 85 90
 Arg Tyr Lys Ala Ala Arg Asn Gln Ala Ala Thr Asn Ala Gln Ile
 95 100 105
 Leu Ala His Val Gly Arg Arg
 110

<210> 11

<211> 38

<212> PRT

<213> *Mus musculus*

<220>

<223> Mouse Urocortin II

<400> 11

Val Ile Leu Ser Leu Asp Val Pro Ile Gly Leu Leu Arg Ile Leu
 5 10 15
 Leu Glu Gln Ala Arg Tyr Lys Ala Ala Arg Asn Gln Ala Ala Thr
 20 25 30
 Asn Ala Gln Ile Leu Ala His Val
 35

<210> 12

<211> 38

<212> PRT

<213> *Takifugu rubripes*

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<223> Pufferfish Urocortin Related Peptide

<400> 12

Leu Thr Leu Ser Leu Asp Val Pro Thr Asn Ile Met Asn Val Leu
5 10 15

Phe Asp Val Ala Lys Ala Lys Asn Leu Arg Ala Lys Ala Ala Glu
20 25 30

Asn Ala Arg Leu Leu Ala His Ile
35

<210> 13

<211> 40

<212> PRT

<213> *Rattus norvegicus*

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<223> Rat Urocortin

<400> 13

Asp Asp Pro Pro Leu Ser Ile Asp Leu Thr Phe His Leu Leu Arg
5 10 15

Thr Leu Leu Glu Leu Ala Arg Thr Gln Ser Gln Arg Glu Arg Ala
20 25 30

Glu Gln Asn Arg Ile Ile Phe Asp Ser Val
35 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/23976

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97/00063 A2 (THE SALK INSTITUTE OF BIOLOGICAL STUDIES) 03 January 1997 (03/01/97), see entire document.	1-46

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 OCTOBER 2001

Date of mailing of the international search report

19 NOV 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 306-3230

Authorized officer

PREMA MERT

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/23976

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C07K 14/485, 14/47, 14/575, 14/695, 16/26; C12N 5/10, 15/12, 15/16, 15/62, 15/63, 15/64; A61K 38/16, 38/17, 38/22, 38/36, 39/395

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

530/350, 399, 402, 403, 387.1, 387.9, 388.1, 388.24; 536/23.1, 23.5, 23.51, 24.3, 24.31; 424/130.1, 139.1, 145.1; 435/69.4, 71.1, 71.2, 471, 320.1, 252.3, 254.11, 325; 514/2, 8, 12;

B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

530/350, 399, 402, 403, 387.1, 387.9, 388.1, 388.24; 536/23.1, 23.5, 23.51, 24.3, 24.31; 424/130.1, 139.1, 145.1; 435/69.4, 71.1, 71.2, 471, 320.1, 252.3, 254.11, 325; 514/2, 8, 12;

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, CAS ONLINE, MEDLINE, CAPLUS

search terms: urocortin II, DNA, polynucleotide, nucleic acid, recombinant production, antibody, treatment, therapy, administration, urocortin-related peptide, conjugate, derivative, fusion, analog, modify